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13. ABSTRACT (Maximum 200 Words)

Preliminary data document that signaling events leading to Ad.mda-7-induced transformed-cell specific apoptosis are tyrosine kinase-independent. These results suggest that mda-7/IL-24 cancer cell-specific activity could occur through mechanisms independent of binding to its currently recognized cognate receptors and might even occur independent of receptor function. An adenovirus vector expressing a non-secreted version of MDA-7/IL-24 protein was generated via deletion of its signal peptide. This non-secreted protein was as effective as wild-type secreted MDA-7/IL-24 in inducing apoptosis in cancer cell lines, and displayed transformed cell specificity and localization of MDA-7/IL-24 in the Golgi/ER compartments. Based on localization as well as signal transduction pathway activation, MDA-7/IL-24 protein appears to induce ER stress that in turn induces proapoptotic events. A new reagent was generated i.e. a bacterially expressed and purified GST-MDA-7 fusion protein. We describe the properties and characteristics of this protein in this report. Treatment of breast cancer cell lines with GST-MDA-7 sensitizes both wild-type and mutant p53 expressing tumor cells to growth inhibitory and antisurvival effects of ionizing radiation. Our results indicate that mda-7/IL-24-mediated apoptosis can be triggered efficiently in the absence of protein secretion and is likely mediated by ER stress. In addition, retention of tumor-specific activity of GST-MDA-7 suggests that this new reagent may also have therapeutic applications and prove to be a useful experimental tool.

14. SUBJECT TERMS
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INTRODUCTION

The objective of the proposed studies focuses on the characterization of a novel tumor cell apoptosis-inducing gene that was identified through the use of subtraction hybridization from human melanoma cells induced to growth arrest and terminally differentiate by treatment with interferon and mezerien. Since expression of this gene correlates with induction of irreversible growth arrest; cancer reversion and terminal differentiation in human melanoma cells it was named melanoma differentiation associated gene-7 (*mda-7*). Additional studies by our group have confirmed its potential gene therapeutic potential in other cancers, including human breast and prostate cancer-derived cells. The elucidation of the mechanistic basis of its selective action will provide valuable insights into ensuring safe use, improving efficacy, suggest potential pharmacological adjuvants or substitutes and possibly give important additional information for developing improved treatment options. Growth suppression and apoptosis was observed when *mda-7*/IL-24 was transfected or transduced into a wide spectrum of human breast cancer cell lines. In contrast, no significant growth inhibitory effect was apparent when this gene was transduced into normal breast human epithelial, endothelial, melanocyte, astrocytes or fibroblast cells. This property of *mda-7*/IL-24 suggests it may have translational potential for the gene-based therapy of breast cancer. Moreover, based on pre-clinical cell culture and animal modeling studies, successful Phase I trials have been performed and a Phase II clinical trial has been recently initiated. *MDA-7*/IL-24 has been delivered to cells, tumor xenografts and patients in clinical trials via a nonreplicating adenovirus (Ad.*mda-7*). These studies are contributing greatly to our understanding of the underlying basis of *mda-7*/IL-24 activity and offers potential for identifying strategies for using small molecule mimetics having equivalent or more potent activity than Ad.*mda-7*. We previously demostrated that *mda-7*/IL-24 cancer cell-specific activity could occur through mechanisms independent of binding to its currently recognized cognate receptors and might even occur independent of receptor function. The present studies were designed to assess whether the potent proapoptotic activity observed with Ad.*mda-7* is due to the intracellular or the secreted *MDA-7*/IL-24 protein. We show that Ad.*mda-7* infection of cancer cells results in activation of ER-stress. We demostrate that *mda-7*/IL-24-mediated apoptosis can be triggered through a intrcellular mechanism (via deletion of the signal peptide of *mda-7/IL-24* sequence) and can occur efficiently in the absence of protein secretion. Also, we report the properties and characteristics of a bacterially expressed and purified GST-MDA-7 fusion protein.

BODY

Task 1. Identify functional domains of *mda-7* relevant to its apoptosis inducing properties. Publications: *Sauane* et al. Cancer Research 64, 2988–2993 (*1*) Gopalkrishnan, R.V., *Sauane*, M., and Fisher, P.B. International Immunopharmacology 4: 635–647 (*1*)

We have shown both by biochemical as well as genetic mutation based criteria that activation of STAT1 and STAT3 by *mda-7*/IL-24 was not essential for the apoptosis triggering activity of the molecule (*2*). Based on that finding, we set out to define the functional domains of *mda-7* and to determine if the apoptotic effect is triggered by intracellular protein, possibly by receptor-independent mechanisms. To this end, an adenovirus vector was constructed that expressed a non-secreted version of *mda-7*/IL-24 lacking the signal peptide (Ad.SP$^-$*mda*-7) (*3*). The extent and modality of killing was compared between the full-length *mda*-7/IL-24 expressing virus (*4*) and Ad.SP$^-$*mda*-7, the results obtained indicated that the effect of Ad.SP$^-$*mda*-7 and Ad.*mda*-7 infection was similar with respect to transformed cell apoptosis induction (*2*) (Fig. 1). MDA-7/IL-24 protein was shown to localize to the ER (*5*)/Golgi compartments (Fig. 2).

Our results and recent studies by others clearly reveal that intracellular *MDA-7*/IL-24 protein is active in inducing transformed cell-specific apoptosis, probably through mechanism involving pathway associated with ER-stress. ER stress is caused by different conditions that perturb ER function. In the particular case of ER-stress caused by misfolded protein accumulation, a highly conserved unfolded protein response (*6*) signal transduction pathway is activated (*5*). The UPR is characterized by the coordinated activation of multiple signal transduction pathways that lead to the suppression of the initiation step of protein synthesis, and trigger the expression of genes encoding ER chaperones, enzymes and structural components of the ER. Prolonged activation of this pathway leads ultimately to apoptosis. Earlier findings from our group support this hypothesis since induction of the GADD genes is classically associated with the stress response including ER-stress pathways. Induction of GADD genes and further upstream events such as activation of p38MAPK were shown to be induced by *mda*-7/IL-24 in a transformed cell-specific manner (*3, 7*). Treatment with Ad.*mda*-7 also specifically activated the p44/42MAPK pathway (*3, 8*). Furthermore, Ad.*mda*-7 infection produced an up-regulation of the inositol 1,4,5-trisphosphate receptor (IP3R) in H1299 cells (*9*). IP3R is an intracellular calcium-release channel implicated in apoptosis and localized in the ER. BiP has been best characterized for its role in protein folding and assembly, and its up-regulation during ER stress is a hallmark of the unfolded protein response (UPR) (*10*). Earlier reports identified putative conserved functional HSP70-like chaperone (BiP)-binding sites in both the helix C and F motifs of MDA-7/IL-24 (*11*). Interestingly, a microarray study was reported showing that *mda-7* is able to induce the expression of ER-stress response genes such as BiP (*12*). Thus an important functional domain mutant, Ad.SP$^-$*mda*-7 was generated and characterized in this funding period. Biochemical studies with this mutant demonstrated the importance of the ER/UPR response in mda-7/IL-24 activity resulting in a high impact publication (*3*). This work has defined other relevant mutagenesis targets, which will be analyzed in year 2.

Task 2. Identify and characterize MDA-7 binding proteins and interacting molecules by generation of a bacterially expressed soluble MDA-7 protein as a GST fusion. Publication: *Sauane* et. al., Oncogene 23, 7679–7690 (2004)

I have initiated Task 2 since progress in this area progressed well for technical reasons. I report for year 1 my findings relating to the properties and characteristics of a bacterially expressed and purified GST-MDA-7 fusion protein. Recent data indicates that GST has high transduction efficiency in various cell types and can be used successfully for intracellular delivery of biologically active peptides (*13*). A GST-MDA-7 fusion protein was expressed and purified from a bacterial expression system to identify and characterize MDA-7 binding proteins and interacting molecules. In the course of these experiments, analysis was also performed to determine if this purified protein had biological activity.

In the context of tumor cell killing, my current recently published findings indicate that bacterially expressed and purified GST-MDA-7 operates in a similar way as does plasmid or adenovirus expressed MDA-7/IL-24 i.e. via JAK/STAT-independent and MAPK-dependent pathways (Figures 3 and 4) (*2, 7*). Furthermore, treatment with GST-MOB-5 (rat orthologue of *mda-7* having ~80% homology (Wang, 2002 #22), purified under the same conditions, did not induce cytotoxic effects in cells and was comparable to treatment with GST protein, indicating that the observed apoptotic effects are not attributed to the novel modifications of the fusion protein but rather are likely a consequence of the activity of the MDA-7/IL-24 moiety of the fusion. The GST-tag might, however, contribute to stability as well as facilitation of protein uptake by cells. There is also a possibility that the uptake process is receptor mediated but the likelihood of currently recognized cognate *mda-7*/IL-24 receptor participation in this process is not very strong. In general, the data shown here indicates that GST-MDA-7 is functionally equivalent to native MDA-7/IL-24 protein with respect to its tumor-killing attributes. Cancer gene therapy using Ad.*mda-7* has significant promise and based on initial successes continues to be evaluated in Phase I/II clinical trials (*4, 6*). The potential use of GST-MDA-7 protein as a therapeutic is intriguing, since it can enlarge the existing MDA-7/IL-24 therapeutic scope to cover tumors resistant to or uninfectable by Ad.*mda-7*. Additional work has demonstrated that both Ad.*mda*-7 as well as the GST-MDA-7 fusion protein radiosensitize primary human glioblastoma cells to comparable extents (*8, 14*). As shown in Figure 5, GST-MDA-7, but not GST, induced a dose-dependent decrease in viability in MDA-MB-231 cells as reflected by MTT assay. MDA-MB-231 cells were treated with bacterially synthesized GST-MDA-7 followed by exposure to ionizing radiation. GST-MDA-7 suppressed MDA-MB-231 cell growth that was enhanced in a greater than an additive fashion by ionizing radiation. The direct growth inhibitory effect of purified protein in additional breast cancer cells was observed when MCF-7, T47D and MDA-MB-157 breast tumor cells were treated with the GST-MDA-7 protein. In contrast, no significant change in viability or growth was observed following treatment of the normal HBL-100 breast epithelial cell lines with GST-MDA-7. When analyzed with anti-GST antibody, both control GST protein as well as GST-MDA-7 is visualized inside cells in extra-nuclear locations. Samples processed in parallel but reacted with anti-MDA-7 antibody show an intracellular localization of GST-MDA-7 protein. Specificity is demonstrated due to lack of detection of protein in GST treated samples reacted with anti-MDA-7 antibody. It therefore appears that cells internalize

GST as well as GST-MDA-7 fusion proteins. It is unclear if this is a receptor-mediated process, although a more likely possibility is that the GST moiety facilitates uptake of protein by cells as recently reported (*13*). Thus in year 1, I have been able to standardize expression and purification conditions, produce and biochemically characterize a novel reagent that might have potential therapeutic potential as well as serve as an important research tool. My initial findings have been published in a high impact journal Oncogene (*15*) and further work in subsequent years will follow up on these initial findings.

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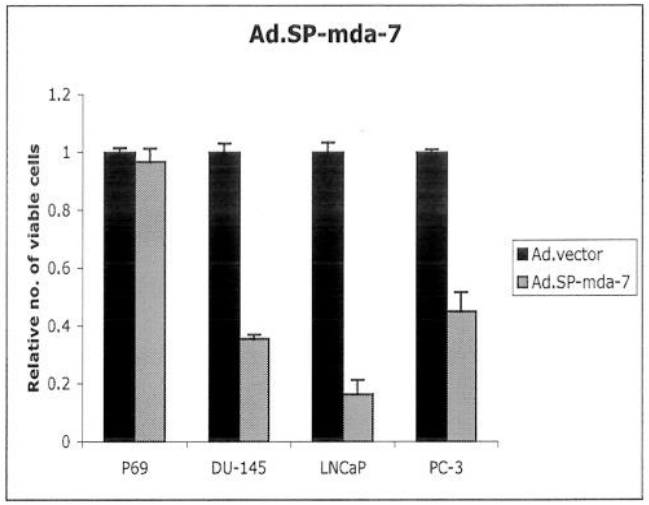
A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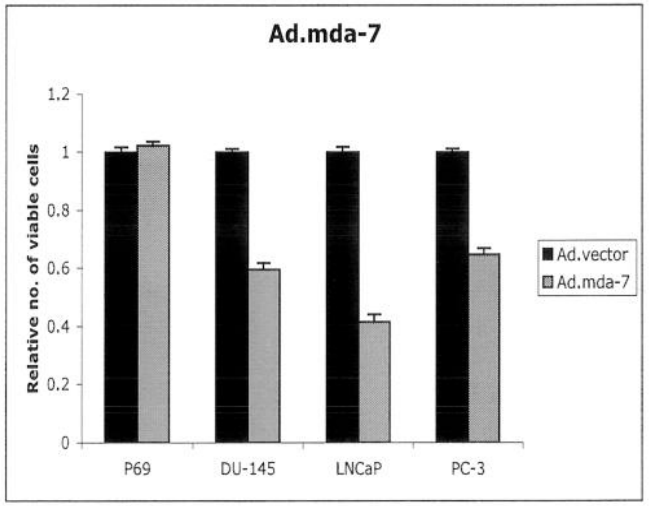
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Relative no. of viable cells
1.2
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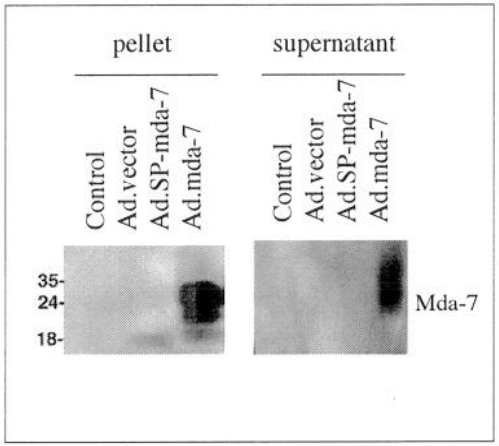
B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supernatant
Control
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Ad.SP-mda-7
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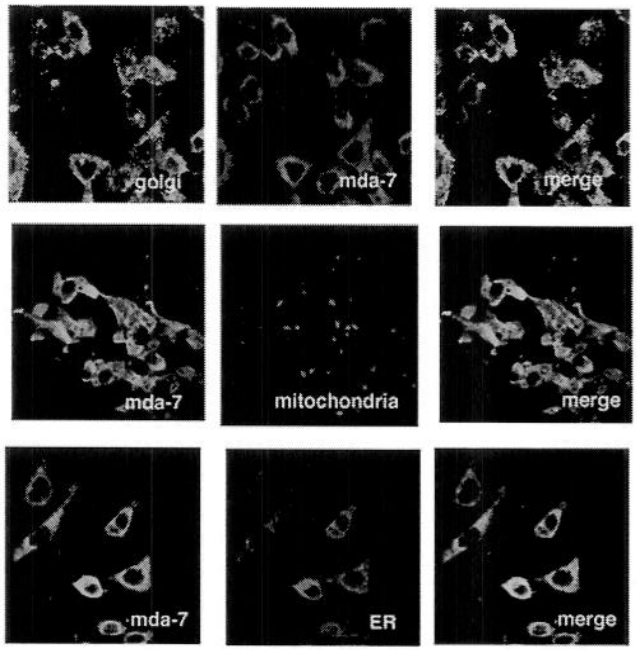
35-
24-
18-
Mda-7

Figure 1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ER
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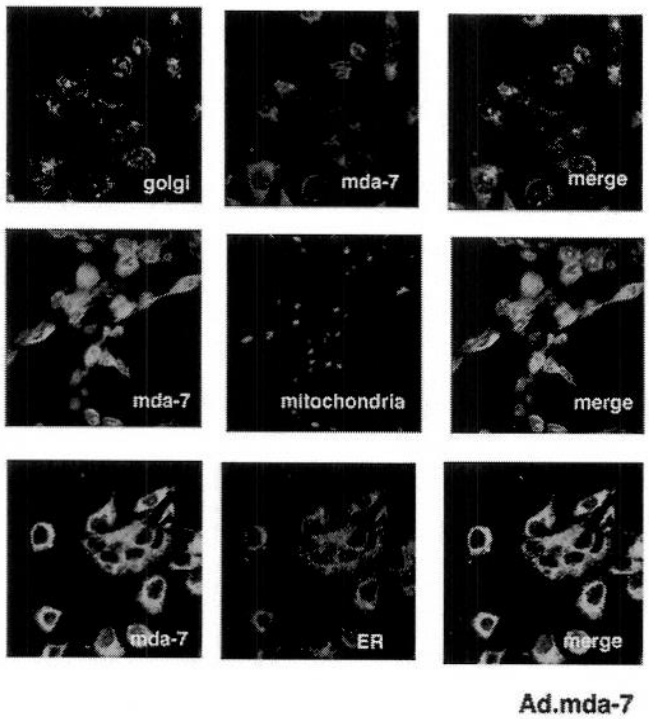
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mda-7
mitochondria
merge
mda-7
ER
merge
Ad.mda-7

figure 2

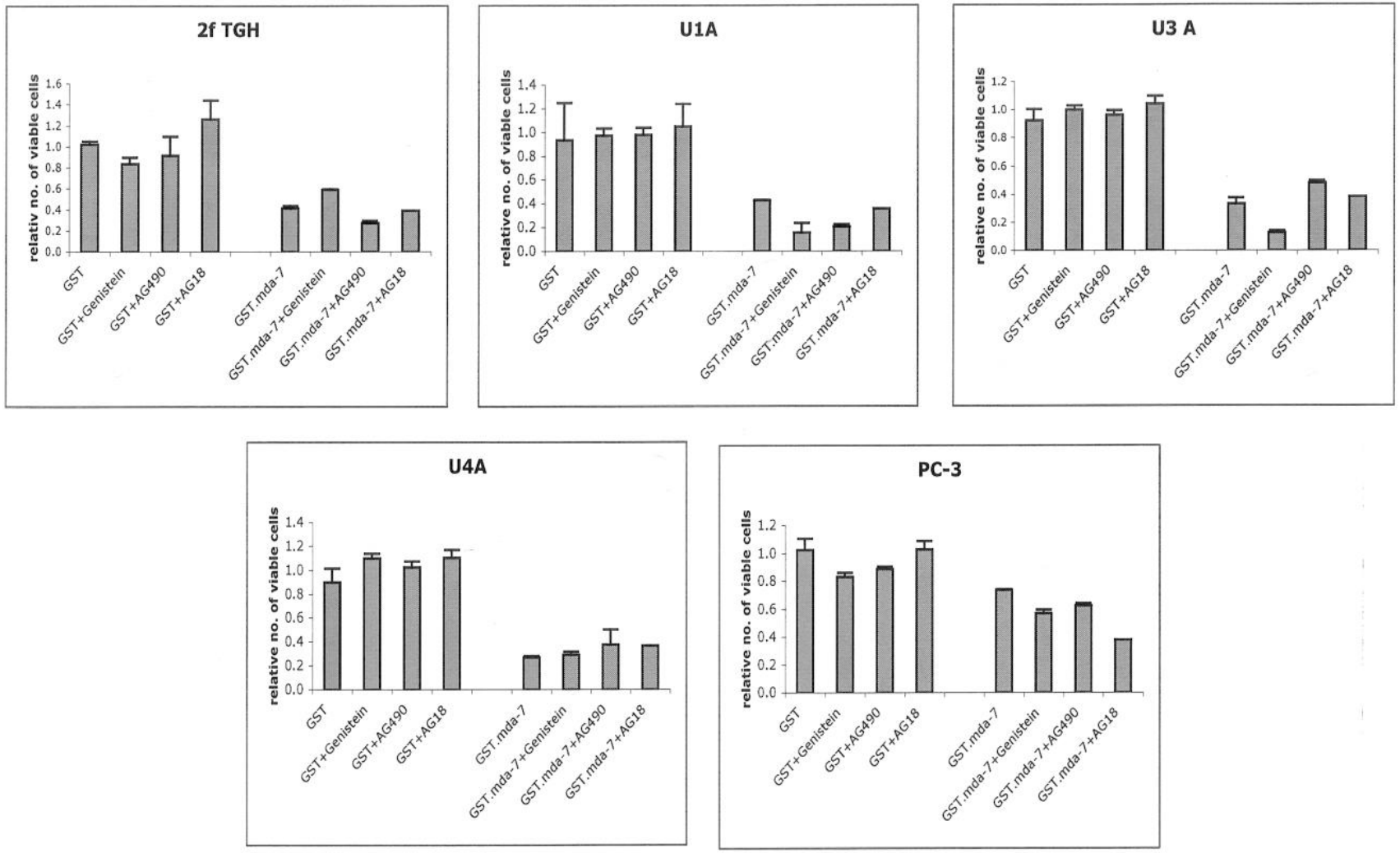
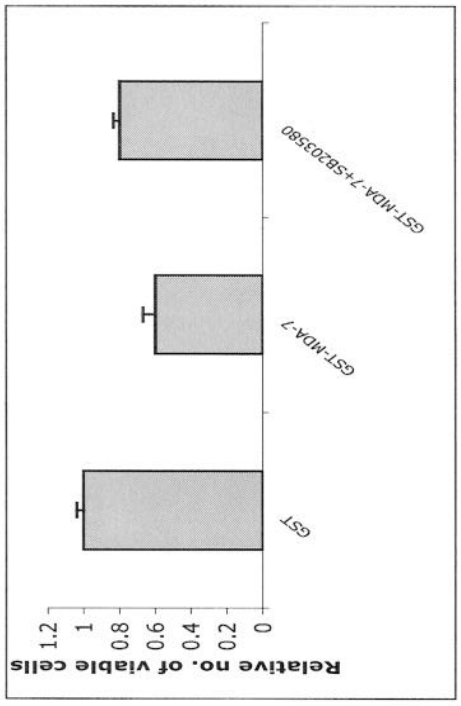

2f TGH
relativ no. of viable cells
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
GST
GST+Genistein
GST+AG490
GST+AG18
GST.mda-7
GST.mda-7+Genistein
GST.mda-7+AG490
GST.mda-7+AG18
U1A
relative no. of viable cells
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
GST
GST+Genistein
GST+AG490
GST+AG18
GST.mda-7
GST.mda-7+Genistein
GST.mda-7+AG490
GST.mda-7+AG18
U3 A
relative no. of viable cells
1.2
1.0
0.8
0.6
0.4
0.2
0.0
GST
GST+Genistein
GST+AG490
GST+AG18
GST.mda-7
GST.mda-7+Genistein
GST.mda-7+AG490
GST.mda-7+AG18
U4A
relative no. of viable cells
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
GST
GST+Genistein
GST+AG490
GST+AG18
GST.mda-7
GST.mda-7+Genistein
GST.mda-7+AG490
GST.mda-7+AG18
PC-3
relative no. of viable cells
1.2
1.0
0.8
0.6
0.4
0.2
0.0
GST
GST+Genistein
GST+AG490
GST+AG18
GST.mda-7
GST.mda-7+Genistein
GST.mda-7+AG490
GST.mda-7+AG18

Figure 3


Relative no. of viable cells
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0.8
0.6
0.4
0.2
0
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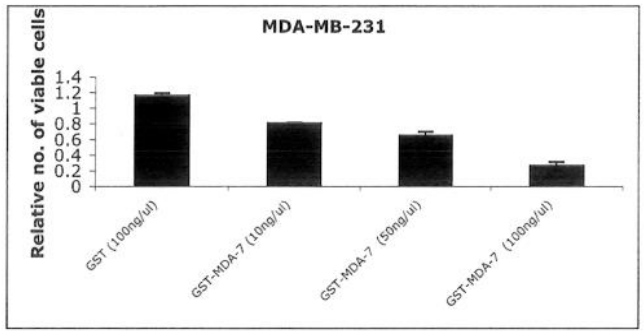
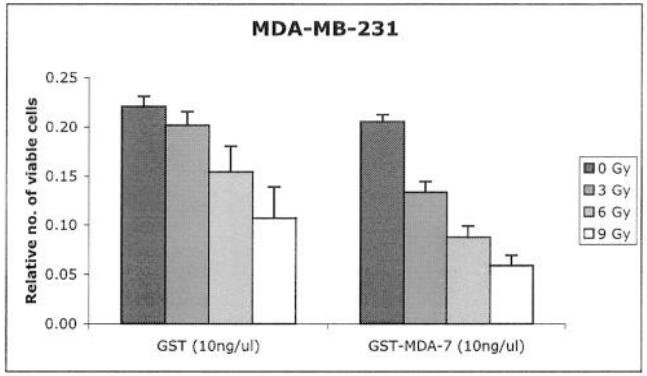
GST-MDA-7
GST-MDA-7+SB203580

Figure 4

A


MDA-MB-231
Relative no. of viable cells
1.4
1.2
1
0.8
0.6
0.4
0.2
0
GST (100ng/ul)
GST-MDA-7 (10ng/ul)
GST-MDA-7 (50ng/ul)
GST-MDA-7 (100ng/ul)

B


MDA-MB-231
Relative no. of viable cells
0.25
0.20
0.15
0.10
0.05
0.00
0 Gy
3 Gy
6 Gy
9 Gy
GST (10ng/ul)
GST-MDA-7 (10ng/ul)

C

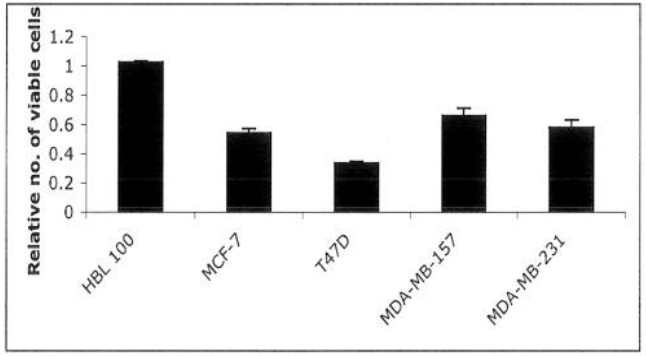

Relative no. of viable cells
1.2
1
0.8
0.6
0.4
0.2
0
HBL 100
MCF-7
T47D
MDA-MB-157
MDA-MB-231

Figure 5

Fig. 1 Comparative growth inhibition, apoptosis induction and MDA-7 expression in cells infected with Ad.*vec*, Ad.*mda*-7 and Ad.SP*mda*-7. A) Growth inhibition in prostate cell lines: Cells were infected with 100 pfu/cell of Ad.*vec*, Ad.SP*mda-7* or Ad.*mda-7* and cell viability was determined by the MTT proliferation assay 5-days after infection. Numbers represent a ratio of specific treatments indicated versus untreated cells. An average of three independent experiments is shown ± S.D. **B) MDA-7/IL-24 expression in DU-145 cells:** Protein lysates were collected from uninfected (control) DU-145 cells and after infection with Ad.*vec*, Ad.*mda*-7 or Ad.SP*mda*-7. Samples (50 mg) were run on 12% SDS-PAGE, transferred to a nitrocellulose membrane and stained with rabbit anti-*mda*-7/IL-24 antibody as described in Materials and methods.

Fig. 2 Localization of the *MDA-7* protein after infection with Ad.SP*mda*-7 or Ad.*mda*-7: MDA-7 protein localization was analyzed by indirect immunofluorescence after infection of DU-145 or P69 cells with 100 pfu/cell of Ad.SP*mda*-7, Ad.*mda*-7, or Ad.*vec* and 48 h post infection cells were fixed and *MDA*-7/IL-24 protein was detected by indirect immunofluorescence using anti-*mda-7*/IL-24 antibody. Images of Golgi, ER and mitochondria were obtained using anti-G130, anti-calreticulin, and MitoTracker, respectively, as described in Materials and methods. Images of the different compartments and *mda*-7/IL-24 were merged. Similar localization of MDA-7/IL-24 protein was observed following infection with the different viruses in P69 cells (data not shown).

Fig. 3 Apoptosis induction by GST-MDA-7 in JAK/STAT deficient human fibrosarcoma cell lines: The indicated cell types were treated with GST or GST-MDA-7 protein. Cells were analyzed for cell viability by MTT assay 5-days later. MTT absorbance of untreated control cells was set at 1 to determine relative number of viable cells. Results are the average of three independent experiments ± S.D.

Fig. 4 Comparative growth inhibition and apoptosis induction in pancreatic cancer cells treated with GST or GST-MDA-7. Cells were treated with GST or GST-MDA-7 and cell viability was determined by the MTT proliferation assay 5-days later. Numbers represent a ratio of specific treatments versus untreated cells. An average of three independent experiments is shown ± S.D.

Fig. 5 Enhanced radiation-induced cell killing in breast cancer cells treated with GST-MDA-7. A) MDA-MB-231 Cells were treated with GST or GST-MDA-7 and cell viability was determined by the MTT proliferation assay 5-days later. Numbers represent a ratio of specific treatments indicated versus untreated cells. **B)** MDA-MB-231 cells were treated with GST-MDA-7 or GST and 24h after, cells were irradiated (3, 6, 9 Gy). Cells were collected 96 h after irradiation and viability was determined by MTT assay. **C)** HBL-100, MDA-MB-157, MDA-MB-231, T47D, and MCF-7 were treated with GST or GST-MDA-7 and cell viability was determined by the MTT proliferation assay 5-days later. Numbers represent a ratio of specific treatments indicated versus untreated cells. An average of three independent experiments is shown ± S.D.

Key Research Accomplishments

- I have been able to demonstrate that intracellular localization to the endoplasmic reticulum (ER) is necessary and sufficient for *mda-7*/IL-24-mediated apoptosis. This unprecedented mode of activity of *mda-7*/IL-24, in the context of its belonging to and displaying all the characteristics of a secreted cytokine, serves to highlight the mechanistic basis of its anti-tumor cell activity. Specifically, we have demonstrated that *mda-7*/IL-24 is able to trigger ER-stress probably due to binding to BiP. This was accomplished by using an adenovirus vector expressing a nonsecreted version of MDA-7/IL-24 protein generated via deletion of its signal peptide.

- I have been able to standardize expression and purification conditions, produce and biochemically characterize a novel reagent that might have potential therapeutic potential as well as serve as an important research tool. GST-MDA-7 retains its cancer-selective apoptosis-inducing properties, thereby providing a new reagent that will assist in defining the mechanism of action of this novel protein. In addition, retention of tumor-specific activity of GST-MDA-7 suggests that this protein may also have therapeutic applications.

Reportable Outcomes:

Sauane, M., et al. (2004). Melanoma differentiation associated gene-7/interleukin-24 promotes tumor cell-specific apoptosis through both secretory and nonsecretory pathways. Cancer Res. 64: 2988 – 2993.

Sauane, M., et al. (2004). Mechanistic aspects of mda-7/IL-24 selectivity analyzed via a bacterial fusion protein. Oncogene, 23: 7679-7690.

Lebedeva, I. Sauane, M. Gopalkrishnan, RV, Sarkar, D; Su, Z-z, Gupta, P., Nemunaitis, J., Cunningham, C, Yacoub, Dent, P., Fisher,P.B. (2004) Mda-7/IL-24: Exploiting Cancer's Achilles' Heel. Molecular Therapy, 11: 4-18.

Conclusions

I have been able to demonstrate that signaling events leading to susceptibility to Ad.*mda-7* or Ad.SP*mda-7*-induced apoptosis have a potent intracellular mode of action and that this molecule is active in inducing transformed cell-specific apoptosis even without secretion. Our results also demonstrate that *mda-7*/IL-24 is able to trigger ER-stress.

I have demonstrated that purified recombinant GST-MDA-7 protein recapitulates the potent tumor suppressing and apoptosis-inducing properties of adenovirally expressed mda-7/IL-24 in breast cancer cells. We thereby provid a new reagent that will assist in defining the mechanism of action of this novel cytokine. In addition, retention of tumor specific activity of GST-MDA-7 suggests that this protein may also have therapeutic applications.

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3. M. Sauane *et al.*, *Cancer Res* **64**, 2988-93 (May 1, 2004).
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Advances in Brief

Melanoma Differentiation Associated Gene-7/Interleukin-24 Promotes Tumor Cell-Specific Apoptosis through Both Secretory and Nonsecretory Pathways

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Abstract

Melanoma differentiation associated gene-7/interleukin-24 (*Mda*-7/IL-24), a novel member of the IL-10 family of cytokines, uniquely displays cancer-specific apoptosis-inducing activity. Positive results in ongoing phase I/II clinical trials have strengthened the possibility of its utilization as a cancer gene therapeutic. Previous studies document that signaling events leading to Ad.*mda*-7-induced transformed cell apoptosis are tyrosine kinase-independent. These results suggest that *mda*-7/IL-24 cancer cell-specific activity could occur through mechanisms independent of binding to its currently recognized cognate receptors and might even occur independent of receptor function. An adenovirus vector expressing a nonsecreted version of MDA-7/IL-24 protein was generated via deletion of its signal peptide. This nonsecreted protein was as effective as wild-type secreted MDA-7/IL-24 in inducing apoptosis in prostate carcinoma cell lines and displayed transformed cell specificity and localization of MDA-7/IL-24 in the Golgi/endoplasmic reticulum compartments. Our results indicate that *mda*-7/IL-24-mediated apoptosis can be triggered through a combination of intracellular as well as secretory mechanisms and can occur efficiently in the absence of protein secretion.

Introduction

Melanoma differentiation associated gene-7 (*mda*-7) was identified by subtraction hybridization in the context of induction of irreversible growth arrest and terminal differentiation of human melanoma cells (1, 2). Transfection of *mda*-7 into a spectrum of human and rodent tumor cells confirmed potent growth-inhibitory properties, not only in the context of melanoma but also in diverse human cancers (3). In contrast, this antigrowth effect was not apparent in normal cells (3). Structural and sequence homology in addition to functional conservation indicated that this gene belongs to the interleukin (IL)-10 family of cytokines and has therefore been redesignated IL-24 (2, 4–9). Several independent studies have demonstrated that a majority of human cancer-derived cell lines, including melanoma, prostate, breast, cervical, lung, fibrosarcoma, pancreatic, colorectal, and glioblastoma undergo apoptosis when exposed to *mda*-7/IL-24 (reviewed in Ref. 6). Current studies indicate that the mechanism by which *mda*-7/IL-24 induces cancer-specific apoptosis-inducing activity is complex, involving multiple signal transduction pathways and intracellular molecules (reviewed in Refs. 6, 10, 11), requiring further clarification.

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The mRNA encoding *mda*-7/IL-24 is ~2 kb and encodes a protein predicted to have a molecular weight of M_r 23,800 (2) belonging to the four-helix bundle family of cytokine molecules (8, 9). The open reading frame encodes a molecule that is 206-amino acids in length, which is a precursor form of the ultimate cleaved, post-translationally processed and secreted mature product. There are three consensus asparagine glycosylation residues that are *N*-glycosylated, resulting in a mature secreted product showing multiple bands on denaturing protein gel electrophoresis likely because of partial and complete sugar modification on available sites (6, 10). The precursor form of *mda*-7/IL-24 is cleaved at position 48, the signal peptidase cleavage recognition site, during import into the endoplasmic reticulum (ER) for processing and secretion via passage through the Golgi apparatus and secretory vesicles. The cleaved unprocessed protein has a predicted molecular weight of M_r 18,200, and several groups have currently demonstrated processing and secretion of the molecule as predicted by the presence of consensus sites relevant to specific processing events (6, 10).

The present studies were designed to assess the relevance of *mda*-7/IL-24 secretion in mediating cancer gene therapy relevant biological effects (*i.e.*, cancer-selective cell killing). Our results confirm that signaling events leading to susceptibility to Ad.*mda*-7- or Ad.SP$^-$*mda*-7-induced apoptosis have a potent intracellular mode of action and that this molecule is active in inducing transformed cell-specific apoptosis even without secretion. Our results also demonstrate significant involvement of extracellular signal-regulated kinase 1/2 (ERK1/2) in *mda*-7/IL-24-induced transformed cell-specific killing as one of several components potentially contributing to this observed activity.

Materials and Methods

Cell Lines, Adenoviruses, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) Viability Assay, Fluorescence-Activated Cell Sorter Analysis, and Cell Counting. All human cancer cell lines were obtained from the American Type Culture Collection (Rockville, MD) other than the human fibrosarcoma 2fTGH and its derivatives, which were a kind gift from Dr. George Stark (Cleveland Clinic, Cleveland, OH). The immortalized normal prostate epithelial cell P69 was provided by Dr. J. Ware (Medical College of Virginia, Richmond, VA). Culture and maintenance of cells and construction, propagation, and utilization of adenoviruses were as described previously (12). Protocols used for MTT, fluorescence-activated cell sorter, and cell counts were as described previously (12).

Western Blot Analyses. Cell lines were grown on 10-cm plates and protein extracts were prepared with radioimmunoprecipitation assay buffer containing a mixture of protease inhibitors. Fifty μg of protein was applied to 12% SDS-PAGE and transferred to nitrocellulose membranes. The membranes were probed with polyclonal antibodies to *mda*-7, phospho-ERK1/2, and total ERK antibodies (12).

Matrigel Invasion Assay. Invasion of C8161 cells *in vitro* was measured as the capacity of cells to pass through a Matrigel-coated transwell insert (Corning Inc., NY). Briefly, transwell inserts with 8-μm pores were coated

with Matrigel (1 mg/ml), cells were trypsinized, and 200-μl aliquots of cell suspension (1×10^6 cells/ml) were added in triplicate wells. After 48-h incubation, cells that passed through the filter into the lower wells were quantified by direct counting.

Bystander Tumor Growth Inhibition Assay. The lower basal layer of cells (P69) was seeded at 2×10^5/6-cm dish and infected at 25 multiplicity of infection with the respective viruses. After 24 h, cells were washed five times with PBS and overlaid with 6 ml of 0.4% Nobel agar containing 1×10^5 DU-145 cells. After 14 days of incubation during which overlay cells were refed every 4 days, macroscopic colonies $\geq$2 mm were scored. Colonies were enumerated from triplicate plates, and values were expressed as an average $\pm$SD.

Immunofluorescence. DU-145 and P69 cells were grown in chamber slides (Falcon; BD Biosciences, San Jose, CA) fixed with 2% paraformaldehyde, permeabilized by 0.1% Triton X-100, and then incubated with primary antibodies (antirabbit *mda-7*), GM130 (BD PharMingen, San Diego, CA), lysosome-associated membrane glycoprotein 1/2 (LAMP1/2; Santa Cruz, CA), calreticulin (BD PharMingen), and Mitrotrack marker (Molecular Probes, Eugene OR). FITC-conjugated donkey antimouse IgG or antirabbit IgG (Molecular Probes) were used for visualization on a Zeiss LSM 510 fluorescence microscope.

Results

Growth Inhibitory Effect of Ad.*mda.7* and *Ad.SP$^-$mda-7* on Prostate Cancer Cell Lines. Experiments were performed to determine whether infection with an adenovirus vector expressing a nonsecreted version of MDA-7/IL-24 protein deleted for the signal peptide (Ad.SP$^-$*mda*-7) could induce growth suppression and apoptosis in prostate tumor cells in a manner analogous to that observed using a full-length *mda*-7/IL-24 (Ad.*mda*-7; Refs. 13, 14). Parallel experiments were performed with a normal immortalized untransformed prostate epithelial cell line (P69; Ref. 15) to define potential differential susceptibility to these viruses. These experiments confirmed that infection with both viruses induced comparable killing in susceptible prostate tumor cell lines (PC-3, DU-145, and LNCaP), but as previously reported, using Ad.*mda*-7 (13) did not affect the viability of P69 cells (Fig. 1*A*).

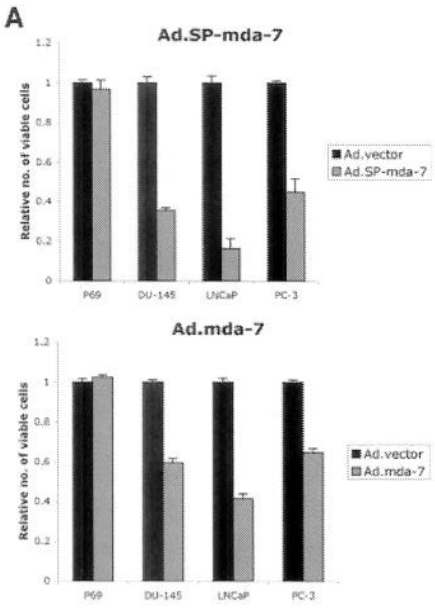
Ad.*mda*-7 or Ad.SP$^-$*mda*-7 infection induced an increase in the proportion of DU-145 cells undergoing apoptosis as reflected by an increase in the proportion of cells with a sub-G_0/G_1 hypodiploid (A_0) DNA content (Fig. 1*B*), as previously described for Ad.*mda*-7 (13). Similar results were obtained when LNCaP or PC-3 prostate tumor cells were infected with the two *mda*-7/IL-24 expressing adenoviruses (data not shown). In contrast, no significant change was observed in the percentage of apoptotic cells after infection of P69 cells with Ad.*vec*, Ad.SP$^-$*mda*-7, or Ad.*mda*-7 (Fig. 1*B*). This data provides further support for equivalent cancer-specific cell killing with Ad.*mda*-7 or Ad.SP$^-$*mda*-7.

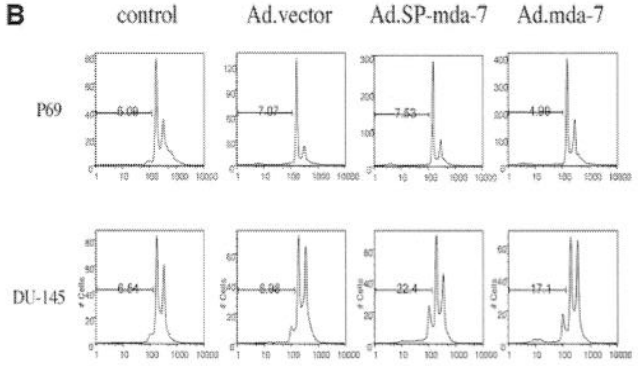
To determine the extent of secretion of MDA-7/IL-24 protein after infection with Ad.SP$^-$*mda*-7 and compare it with wild-type Ad.*mda*-7, we analyzed the supernatant and pellets of infected cells by Western blotting 24 h after infection (Fig. 1*C*). Intracellular protein was observed in DU-145 cells in extracts derived from both Ad.SP$^-$*mda*-7 and Ad.*mda*-7. Secreted MDA-7/IL-24 protein was found in the supernatants only from Ad.*mda*-7-infected cell lines at 24 h (as well as 48 and 72 h; data not shown). The intracellular fractions of *mda*-7/IL-24 expressed by Ad.SP$^-$*mda*-7 differed from wild-type Ad.*mda*-7-expressed protein in that the only band present in both extracts was a lower molecular weight band of $\sim M_r$ 18,000. The additional higher molecular weight bands seen in the intracellular Ad.*mda*-7 lane (Fig. 1*C*) are likely the previously reported post-translationally processed forms of this molecule (16–18). This strengthens the possibility that absence of signal peptide impacts on further post-translational processing of the mutant protein, including lack of secretion. Because in both cases the killing effect is comparable, it is possible that the active form of protein does not necessarily require processing but might need localization to ER and Golgi compartments of cells to be functional. We have also noted an apparently lower amount of MDA-7/IL-24 protein expression, by Western blotting (Fig. 1*C*, *left panel*) with the Ad.SP$^-$*mda*-7 virus compared with Ad.*mda*-7. The viral titers used in these studies are equivalent, as is the extent of cell killing (Fig. 1, *A* and *B*). It is possible that (*a*) stronger intensity generated by glycosylated protein bands attributable to additional antibody trapping of primary or secondary antibody on sugar residues produces an apparently stronger signal for a given amount of protein compared with unglycosylated molecules (*b*) lower stability of unglycosylated MDA-7 protein because the sugar modification might contribute to stability results in an overall lower steady-state level of this form of protein. The amount of *mda*-7/IL-24 mRNA expressed by both viruses is comparable in Northern blot analyses.[5] Regardless, the phenotypic effect of these two viruses irrespective of the apparent differences in protein expression level is essentially identical with respect to growth inhibition and apoptosis induction.

We previously demonstrated activation of ERK1/2 in glioblastoma cells upon Ad.*mda*-7 infection (19). To define whether ERK1/2 activation also plays a role in *mda*-7/IL-24-induced killing in prostate cancer cell lines we used PD98059, a specific mitogen-activated protein kinase kinase 1 signal pathway inhibitor (19). This pharmacological agent inhibited killing of DU-145 cells to a comparable extent after infection with either Ad.SP$^-$*mda*-7 or Ad.*mda*-7, whereas a similar experimental protocol did not affect the viability of P69 cells (Fig. 1*D*). A similar inhibition in cell killing was also apparent in PD98059-treated LNCaP and PC-3 cells infected with both viruses (data not shown). To further substantiate this observation, lysates of P69 and DU-145 cells, either uninfected or infected with the Ad.SP$^-$*mda*-7 or Ad.*mda*-7 virus, were analyzed by SDS-PAGE followed by Western blotting with antiphospho-ERK1/2 and anti-ERK (total) antibodies. As shown in Fig. 1*D*, treatment with Ad.SP$^-$*mda*-7 or Ad.*mda*-7 promoted ERK1/2 phosphorylation in prostate cancer cell lines, but not in the P69 cell line, correlating cell killing with activation of the ERK1/2 pathway. As documented previously for Ad.*mda*-7 (12), we observed that the Ad.SP$^-$*mda*-7 virus was capable of inducing apoptosis in cells functionally deficient for Janus-activated kinase/signal transducers and activators of transcription (JAK/STAT) signaling (Ref. 20; data not shown), further indicating functional equivalence.

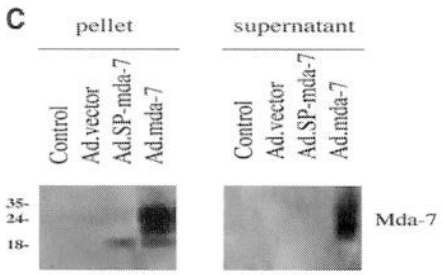
Secreted and Nonsecreted Forms of *mda*-7/IL-24 Inhibit Tumor Cell Invasion. An additional comparison of the relative potencies of both forms of MDA-7/IL-24 protein focused on their impact on tumor cell invasiveness. For this analysis, the effect of Ad.SP$^-$*mda*-7 and Ad.*mda*-7 infection on the invasiveness of C8161 cells (metastatic human melanoma cells) was studied. This cell line was chosen because of its well-documented and reproducible invasive capacity *in vitro* as well as its tumorigenic and metastatic properties in *in vivo* nude mouse assays (21, 22). Invasiveness was evaluated using a Matrigel-basement membrane model that determines the invasion of infected cells through a layer of Matrigel-coated 8.0-μm pore size tissue culture inserts. Infection of C8161 cells with Ad.SP$^-$*mda*-7 or Ad.*mda*-7 inhibited to a similar extent the ability of these cells to invade through Matrigel-coated membrane inserts as compared with Ad.*vec*-infected cells (Fig. 2*A*, *top panel*). This effect, which was apparent 48 h after infection with the MDA-7/IL-24 expressing adenoviruses, occurred without any apparent effect on C8161 cell

[5] M. Sauane and P. B. Fisher, unpublished data.





	control	Ad.vector	Ad.SP-mda-7	Ad.mda-7
P69	6.09	7.07	7.53	4.99
DU-145	6.54	8.98	22.4	17.1



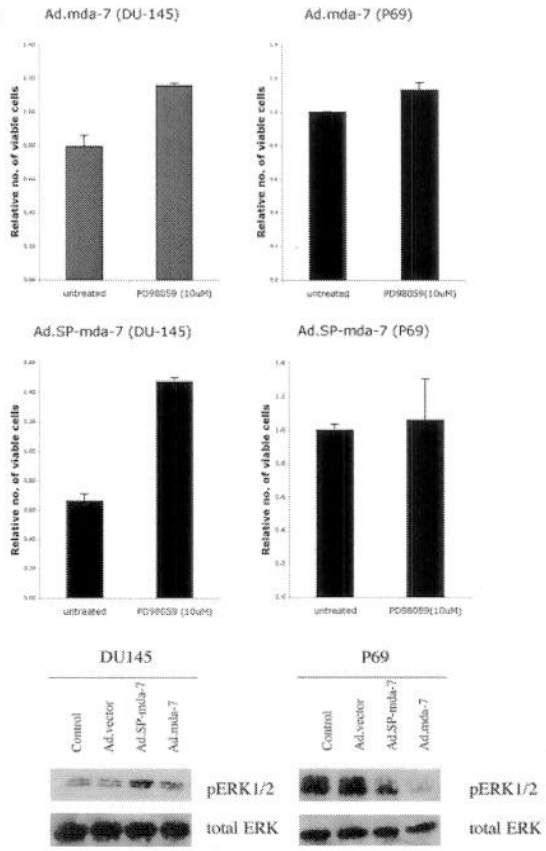


Fig. 1. Comparative growth inhibition, apoptosis induction, and MDA-7 expression in cells infected with Ad.*vec*, Ad.*mda*-7, and Ad.SP^{-}*mda*-7. *A*, growth inhibition in prostate cell lines. Cells were infected with 100 pfu/cell of Ad.*vec*, Ad.SP^{-}*mda*-7, or Ad.*mda*-7, and cell viability was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide proliferation assay 5 days after infection. *Numbers* represent a ratio of specific treatments indicated *versus* untreated cells. An average of three independent experiments is shown ±SD *B*, apoptosis induction in prostate cancer cell lines. DU-145 and P69 cells were treated as described (in *A*), and the percentage of the cells in the A_0 fraction (M1 bars) was determined 24 h later by fixation and staining with propidium iodide and quantitating by fluorescence-activated cell sorter analysis using the CellQuest software (Becton Dickinson) as described in "Materials and Methods." *C*, MDA-7/IL-24 expression in DU-145 cells. Protein lysates were collected from uninfected (control) DU-145 cells and after infection with Ad.*vec*, Ad.*mda*-7, or Ad.SP^{-}*mda*-7. Samples (50 μg) were run on 12% SDS-PAGE, transferred to a nitrocellulose membrane, and stained with rabbit anti-*mda*-7/IL-24 antibody as described in "Materials and Methods." *D*, effect of ERK1/2 inhibitor on *mda*-7/IL-24-induced killing in prostate cancer cell lines. *Top panel*, cells were incubated in the absence or presence of PD98059 (10 μm) after infection with 100 pfu/cell of Ad.*vec*, Ad.SP^{-}*mda*-7, or Ad.*mda*-7. Cell viability was determined by MTT assay 6 days after infection. MTT absorbance of untreated control cells was set at 1 to determine relative number of viable cells. Results shown are an average of three independent experiments; *bars*, ±SD ERK1/2 induction after *mda*-7 expression in prostate cancer cell lines. *Bottom panel*, activation of the ERK1/2 pathway was determined by Western blotting using phospho-specific antibody 24 h after infection with 100 pfu/cell of the different viruses. *pfu*, plaque-forming unit; *IL*, interleukin; *ERK*, extracellular signal-regulated kinase.

growth in monolayer culture (Fig. 2*A*, *bottom panel*), thereby confirming that invasiveness was not inhibited because of loss of cell viability. These results show that both constructs inhibit invasion with equivalent potency, providing yet another illustration of similar biological activity of these molecules.

Only the Secreted Form of *mda*-7/IL-24 Displays "Bystander" Antitumor Activity. Earlier studies in pancreatic cancer cells indicated that MDA-7/IL-24 protein possessed a potent bystander killing activity that exerted growth suppressive and apoptotic effects on nontransduced neighboring tumor cells (23, 24). To determine the extent of bystander

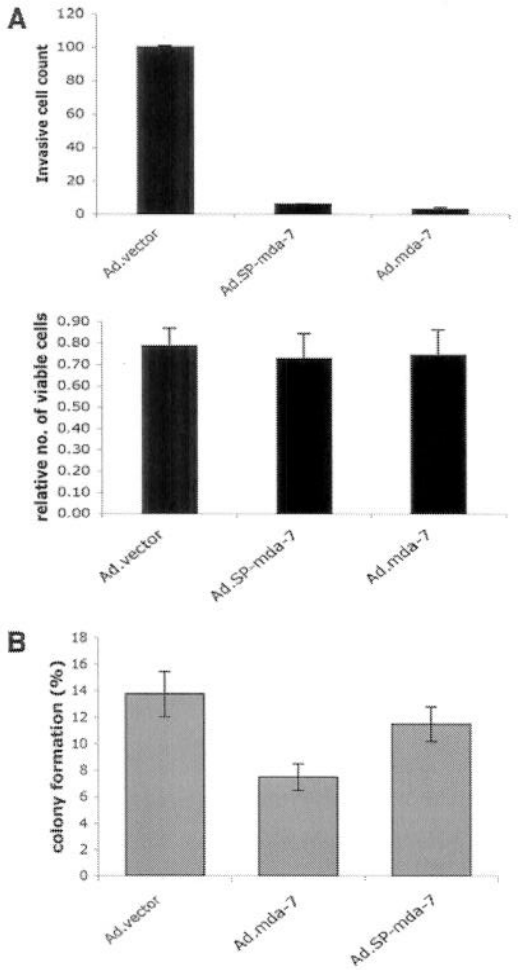


Fig. 2. Comparative mechanism of *mda*-7/IL-24 action after infection of various cell lines with Ad.*vec*, Ad.*mda*-7, and Ad.SP^{-}*mda*-7. *A*, *mda*-7/IL-24 inhibits C8161 Matrigel invasiveness without altering C8161 viability. C8161 cells were infected with 100 pfu/cell of Ad.*vec*, Ad.*mda*-7, or Ad.SP^{-}*mda*-7. After 24 h, 1×10^6 cells were allowed to invade for 48 h through transwell inserts (8-μm pores) coated with Matrigel. The cells that invaded through the Matrigel-coated inserts were stained, counted, and photographed under a light microscope at ×20 magnification. Cells viability was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay in parallel to ascertain whether the inhibition of invasion was associated with a decrease in growth or viability of infected C8161 cells, *bars*, ±SD. Direct cell counts were performed on all surviving, attached cells in the lower chamber to quantitate the relative efficiency of invasiveness. *B*, "bystander" suppression of anchorage-independent growth of DU-145 cells after adenovirus infection of P69 cells. P69 cells were seeded at 2×10^5/6-cm plate, infected 24 h later with 25 pfu/cell of Ad.*vec*, Ad.*mda*-7, or Ad.SP^{-}*mda*-7 and overlaid with 1×10^5 DU-145 cells suspended in 0.4% agar. Fourteen days later, with agar medium feeding every 4 days, the number of anchorage-independent DU-145 colonies ≥2 mm was enumerated microscopically. Average number of colonies ±SD from triplicate plates. Qualitatively similar results were obtained in two additional studies. *pfu*, plaque-forming unit; *IL*, interleukin.

activity, if any, by the nonsecreted mutant form of the protein *versus* the secreted form of MDA-7/IL-24, a dual cell culture agar overlay approach was used. For this protocol, P69 cells that are resistant to killing by MDA-7/IL-24, although serving as a source of production of this cytokine, were infected with the different viruses followed by overlaying with agar containing susceptible DU-145 cells (Fig. 2*B*). Using this strategy, infection of P69 cells with Ad.*mda*-7 (25 pfu/cell) resulted in a reduction in both the number and size of DU-145 colonies growing in agar. In contrast, infection of P69 cells with Ad.SP^{-}*mda*-7 did not induce a significant alteration in DU-145 anchorage independence nor did it decrease the size of colonies growing in the agar overlay as compared with cells infected with Ad.*vec*. These studies provide direct support for a role of secreted MDA-7/IL-24 in mediating a "bystander" cancer growth-inhibitory effect.

Localization of MDA-7/IL-24 to ER/Golgi Compartments. In view of comparable apoptotic induction obtained with Ad.SP^{-}*mda*-7 *versus* Ad.*mda*-7, it was important to determine the location of the signal peptide-deleted MDA-7/IL-24 protein. Therefore, comparative subcellular localization of MDA-7/IL-24 protein was analyzed in DU-145 and P69 cells after infection with the Ad.SP^{-}*mda*-7 and Ad.*mda*-7 viruses. In these experiments, immunofluorescence detection was standardized at different time points to determine whether postinfection time-dependent changes in localization occurred. We also tried to avoid potentially misleading changes in localization that might occur as a result of the loss of internal membrane integrity because of apoptotic events induced by *mda*-7/IL-24. Comparison of the immunofluorescence data using different batches of viruses, cells and secondary antibodies performed at independent times, yielded similar reproducible patterns of staining with both viruses; representative data are presented for DU-145 in Fig. 3. Similar localization results were seen with P69 cells (data not shown). MDA-7/IL-24 protein was detected only in extra-nuclear regions of individual cells. Although there was a light background cytoplasmic staining, protein location primarily overlapped that of the ER stained with anticalreticulin (Fig. 3). The colocalization of MDA-7/IL-24 in Golgi apparatus was also detected via colocalization with anti-GM130 staining (Ref. 25; Fig. 3). However, no co-localization of MDA-7/IL-24 in mitochondria labeled with MitoTracker red occurred (Ref. 26; Fig. 3) confirming staining specificity.

Discussion

On the basis of a number of factors, including its abilities to selectively induce apoptosis in a large spectrum of human cancer-derived cell lines without harming normal cells (reviewed in Ref. 6), inhibitory effects on the growth of human cancer cell xenografts in nude mice (23, 27), and most importantly its capacity to induce tumor regression after intratumoral injection in human tumors in currently ongoing clinical trials (28, 29), the likelihood of *mda*-7/IL-24 becoming a mainstream cancer gene therapeutic appears highly probable (6, 30). Consequently, considerable interest now exists in elucidating the mechanism by which *mda*-7/IL-24 distinguishes between normal and transformed cells. Just how *mda*-7/IL-24 induces this selective effect is clearly very complex, as underscored by experiments described in this manuscript and elsewhere (12) showing that the molecule can function independently of JAK/STAT signal transduction pathways that are classically involved in cytokine-mediated activities. We have additionally demonstrated by sensitive reverse transcription-PCR methodology that apoptosis can be induced in tumor cells not expressing detectable levels of IL-20/IL-22 receptors that bind to MDA-7/IL-24 (12).

The next logical step in pursuing our initial findings of JAK/STAT independence and potential lack of requirement of receptor binding for the antitransformed cell activity of *mda*-7/IL-24 (12) was to determine whether the apoptotic effect could be triggered by intracellular fractions (possibly by receptor-independent mechanisms) or if extracellular MDA-7/IL-24 protein (receptor mediated) was mandatory for activity. To achieve this objective, an adenovirus vector was constructed that expresses a nonsecreted version of MDA-7/IL-24 protein by deleting the 48 amino acid signal peptide, and the extent of killing, signal transduction pathway activation, intracellular localization, invasiveness, and bystander growth-inhibitory activity was compared with the full-length *mda*-7/IL-24. Although most of the analyses

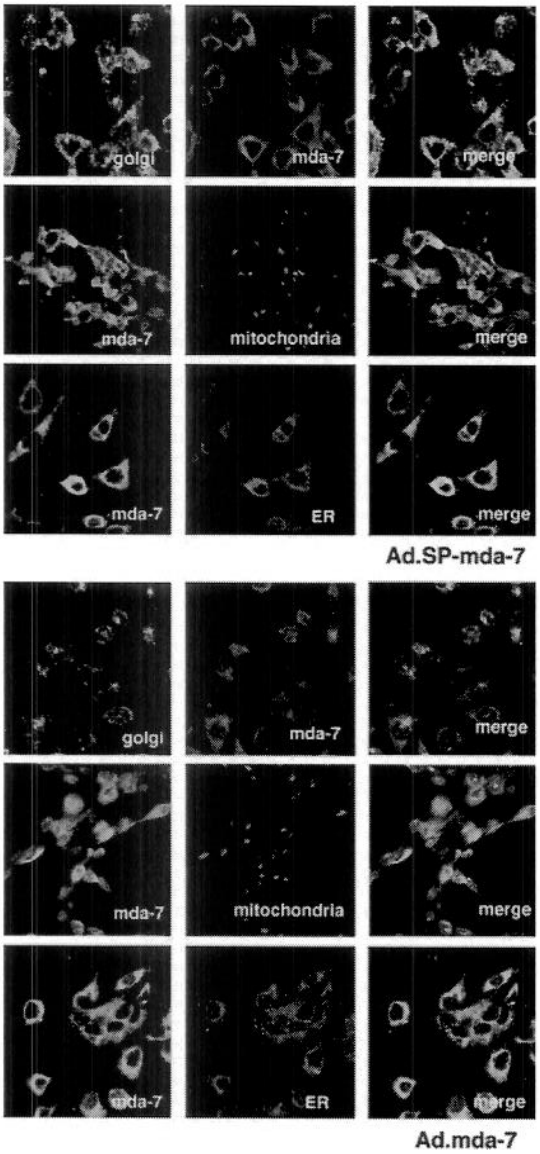


Fig. 3. Localization of the *MDA-7* protein after infection with Ad.SP$^-$*mda-7*, or Ad.*mda-7*. MDA-7 protein localization was analyzed by indirect immunofluorescence after infection of DU-145 or P69 cells with 100 pfu/cell of Ad.SP$^-$*mda-7*, Ad.*mda-7*, or Ad.*vec*, and 48 h postinfection cells were fixed, and *MDA-7*/IL-24 protein was detected by indirect immunofluorescence using anti-*mda-7*/IL-24 antibody. Images of Golgi, ER, and mitochondria were obtained using anti-G130, anticalreticulin, and MitoTracker, respectively, as described in "Materials and Methods." Images of the different compartments and *mda-7*/IL-24 were merged. Similar localization of MDA-7/IL-24 protein was observed after infection with the different viruses in P69 cells (data not shown). *IL*, interleukin; *ER*, endoplasmic reticulum.

were performed in human prostate cell lines, additional studies[5] using a spectrum of cancer and normal cell lines, suggest that these observations are equally applicable to other human cancers.

The results presented here provide several independent lines of evidence indicating that the effect of Ad.SP$^-$*mda-7* and Ad.*mda-7* infection is similar with respect to transformed cell apoptosis induction. In particular, treatment of susceptible prostate cancer cell lines with Ad.*mda-7* as well as Ad.SP$^-$*mda-7* induces killing to a comparable extent through ERK1/2-dependent and JAK/STAT-independent pathways. The fact that both secreted and nonsecreted forms of MDA-7/IL-24 protein have comparable apoptosis-inducing activity was unanticipated, adding a further level of complexity in understanding how this novel molecule works. Localization of full-length MDA-7/IL-24 protein in the ER/Golgi compartments is consistent with the signal peptide hypothesis (31) and the currently known and predicted secreted cytokine nature of the protein (6, 10). Because the signal-peptideless mutant MDA-7/IL-24 protein does not contain an export signal, it is predicted to remain in the cytosol. We have, however, confirmed through confocal immunofluorescence studies that a significant fraction of this protein is able to enter the ER and Golgi apparatus and that proteins derived from wild-type and mutant virus appear to have overlapping patterns of localization within the cell. It is not possible to rule out cryptic internalization signals that become active in the absence of the actual signal peptide, identity of these cryptic sites being currently unknown. Western blot analyses performed on protein-derived cytosolic and extracellular fractions of cells infected with both viruses indicate that only full-length MDA-7/IL-24 is processed and secreted. It is also possible that adenovirus infection produces relatively large amounts of protein that even in the absence of a specific targeting sequence possesses the ability to cross membranes and accumulate in ER/Golgi because of charge and/or tertiary structure. However, because localization of MDA-7/IL-24 is similar in both normal (P69; data not shown) and cancer (DU-145; Fig. 3) cells, differences in cellular localization of this protein can be excluded as a direct mechanism underlying the differential apoptosis-inducing activity of MDA-7/IL-24 toward cancer cells.

From the mechanistic, apoptosis-induction standpoint, programmed cell death pathways are activated by a diverse array of cell extrinsic and intrinsic signals, most of which are ultimately coupled to an obligatory signal propagation event mediated through mitochondria. In the context of localization of MDA-7/IL-24 to the ER/Golgi, emerging evidence suggests that the ER also regulates apoptosis both by sensitizing mitochondria to a variety of extrinsic and intrinsic death stimuli and by initiating cell death signals of its own (32, 33). The observations presented here, raise the question, based on its apparent propensity for ER localization, whether MDA-7/IL-24 protein induces a recently recognized phenomenon of "ER-stress" that in turn induces proapoptotic events (32, 33). Earlier findings from our group support this hypothesis because induction of the GADD genes is classically associated with the stress response including ER-stress pathways. Induction of GADD genes and further upstream events such as activation of p38 mitogen-activated protein kinase was shown to be induced in a transformed cell-specific manner after Ad.*mda-7* infection (34). In addition, we show in the present report that both viruses only in the context of transformed cells also specifically activate the p44/42 mitogen-activated protein kinase pathway. Furthermore, Ad.*mda-7* infection produced an up-regulation in inositol 1,4,5-trisphosphate receptor in H1299 cells (35). Inositol 1,4,5-trisphosphate receptor is an intracellular calcium-release channel implicated in apoptosis and localized in the ER. Whereas further investigations to determine the mechanism of specificity of MDA-7/IL-24-triggered ER-stress are clearly needed, this report for the first time identifies the existence of a cellular ER-stress mechanism that can be differentially activated in transformed cells by MDA-7/IL-24 and possibly other agents. This finding uncovers a new intracellular locus that may prove amenable for potential cancer therapeutic targeting.

Taken together, our results indicate that *mda-7*/IL-24-mediated apoptosis can be triggered through intracellular localization as well as via secretion, and in contexts where both are present, a combinatorial effect is probable. Our results, outlined in a model (Fig. 4), clearly reveal that nonsecreted intracellular MDA-7/IL-24 is also active in inducing transformed cell-specific apoptosis, probably through mechanisms mediated by the signaling pathways transduced through the

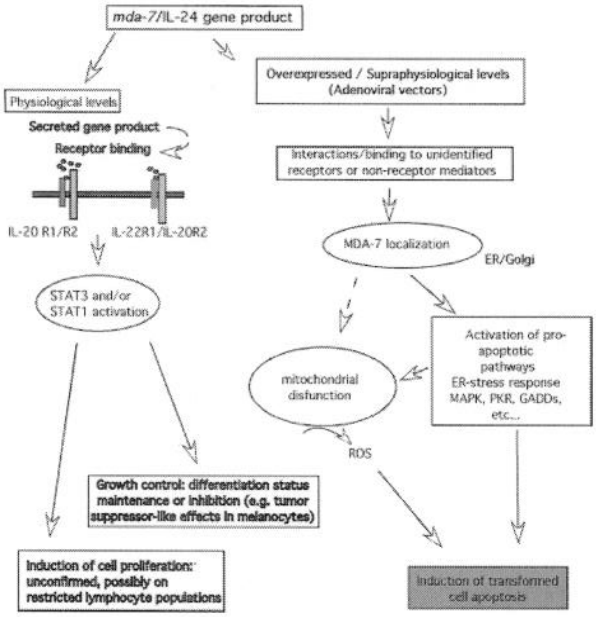


Fig. 4. Model illustrating the possible molecular basis of mda-7/IL-24 cancer cell-mediated apoptosis. The effects of known physiological and ectopic overexpression of *mda*-7/IL-24 are shown on *left* and *right sides of the figure*, respectively. Normally, *mda*-7/IL-24 binds to cognate receptors and activates STAT-1 and -3 transcription factors to mediate pathways affecting cell growth. Because *mda*-7/IL-24 mRNA and protein are normally seen in subpopulations of immune cells and melanocytes, effects are likely initiated in these cell types but might also affect neighboring nonproducing cells because the protein is secreted. When normally or ectopically overexpressed, current findings in this report indicate localization to the ER/Golgi compartments, whether or not the protein contains a secretory signal. Accumulation of MDA-7/IL-24 protein in this compartment triggers apoptosis that could apparently involve induction of pathways described currently as ER-stress. However, MDA-7/IL-24 additionally acts indirectly on mitochondria to generate reactive oxygen species. A combination of pathways triggered by *mda*-7/IL-24 results in transformed cell-specific apoptosis. *IL*, interleukin; *ER*, endoplasmic reticulum; *STAT*, signal transducers and activators of transcription; *ROS*, reactive oxygen species.

ER and Golgi compartments. These newer findings are provocative, although enigmatic, and indicate that much still remains to be learned about the mechanism of action of *mda*-7/IL-24, both in relation to its cancer-selective killing properties and to its potential immune modulatory functions (6, 30). However, based on the initial successes of this cytokine in phase I/II clinical studies in solid cancers and melanomas (28), this effort is certainly justified and holds promise for developing ways of enhancing the clinical utility of this novel cancer-gene therapeutic in treating diverse human neoplasms (6, 36).

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Mechanistic aspects of *mda-7*/IL-24 cancer cell selectivity analysed via a bacterial fusion protein

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The human *mda-7*/IL-24 gene product is normally expressed in melanocytes and certain lymphocyte populations. Loss of expression, a distinctive feature of many tumor suppressor genes, has been documented at RNA and protein levels in association with melanoma progression both *in vitro* as well as in human tumor-derived material. The MDA-7/IL-24 protein undergoes post-translational processing, including removal of an amino-terminal 48-residue signal peptide and extensive glycosylation prior to its secretion by producing cells. Its inherent cytokine properties have been documented in multiple reports, which have identified and characterized its cognate receptors and activation of the JAK/STAT signaling pathway following ligand/receptor docking. A notable and incompletely understood property of MDA-7/IL-24 is its ability to induce apoptosis in transformed cells, while having marginal growth suppressive effects on normal primary or immortalized cell lines. MDA-7/IL-24 has been delivered to cells, tumor xenografts and patients in clinical trials via a nonreplicating adenovirus (Ad.*mda-7*). Studies utilizing eukaryotically expressed and purified MDA-7/IL-24 protein from several sources have recapitulated some of the molecule's reported properties including receptor binding and JAK/STAT activation. Here, we report the properties and characteristics of a bacterially expressed and purified GST-MDA-7 fusion protein. These studies reveal that GST-MDA-7 retains its cancer-selective apoptosis-inducing properties, thereby providing a new reagent that will assist in defining the mechanism of action of this novel cytokine. In addition, retention of tumor-specific activity of GST-MDA-7 suggests that this protein may also have therapeutic applications.
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Introduction

mda-7/IL-24 displays all the characteristics of a typical cytokine including secretion, receptor binding, activation of JAK/STAT signaling and modulation of growth characteristics of responsive cells (Huang *et al*., 2001; Caudell *et al*., 2002; Sauane *et al*., 2003b). This molecule was initially discovered using a differential gene expression subtraction screening strategy to identify and clone genes upregulated during melanoma cell differentiation (Jiang and Fisher, 1993; Jiang *et al*., 1995). Owing to no obvious homology to sequences in public databases, it was initially not apparent that *mda-7* was a cytokine-related molecule. Currently, in addition to biochemical data demonstrating secretion and cytokine properties, location of *mda-7*/IL-24 in an IL-10 family-related genomic cluster on human chromosome 1q22/1q23 has emphasized its actual functional identity (Blumberg *et al*., 2001; Huang *et al*., 2001). Thus, the recently recognized IL-10 family comprises six members including IL-10, IL-19, IL-20, IL-22, mda-7/IL-24 and IL-26 (Gallagher *et al*., 2000; Blumberg *et al*., 2001; Pestka *et al*., 2004), none of which share significant homology at the primary amino-acid level, but which clearly possess functional and structural conservation justifying their subclassification as a distinct cytokine subfamily. Recent work has focused on determining the actual physiological roles of each molecule and the extent of their functional overlap or distinctiveness (Caudell *et al*., 2002; Fickenscher *et al*., 2002; Kisseleva *et al*., 2002; Kotenko, 2002; Parrish-Novak *et al*., 2002; Sarkar *et al*., 2002a; Pestka *et al*., 2003, 2004; Sauane *et al*., 2003b).

A unique property of *mda-7*/IL-24 is its ability to induce apoptosis specifically in transformed cells while having no apparent harmful effect on normal counterparts, including normal human tissues in *in vivo* clinical trial contexts (Jiang *et al*., 1996a; Su *et al*., 1998; Madireddi *et al*., 2000; Saeki *et al*., 2000; Chada *et al*., 2001; Huang *et al*., 2001; Lebedeva *et al*., 2002; Sarkar *et al*., 2002b; Fisher *et al*., 2003; Nemunitais, 2003). This unique property is under intense scrutiny from the viewpoint of mechanism of tumor cell specificity to more effectively translate *mda-7*/IL-24 from the laboratory into the clinic as a cancer gene therapeutic (Chada *et al*., 2001; Fisher *et al*., 2003;

Nemunitais, 2003). Various reagents have been raised to address the functional activity and transformed cell-specificity of *mda*-7/IL-24 including an adenovirus (Jiang *et al*., 1996b) used in clinical trails as well as plasmid expression vectors (Jiang *et al*., 1995, 1996b), antibodies and various forms of purified protein utilized in the laboratory (Caudell *et al*., 2002; Parrish-Novak *et al*., 2002; Ramesh *et al*., 2003; Yacoub *et al*., 2003a, 2004).

Schistosoma japonicum derived Glutathione-*S*-transferase (GST)-tagged fusion proteins have several favorable attributes that facilitate protein expression and purification that are not achievable for proteins expressed in untagged native form (Smith and Johnson, 1988; Zhan *et al*., 2001). Fusions can be easily expressed in the *Escherichia coli* system with high yield and this approach has been effective for a wide range of biologically active peptides or proteins, without disrupting their native activity (Zhan *et al*., 2001). Recent data indicate that GST has high transduction efficiency in various cell types and can be used successfully for intracellular delivery of biologically active peptides (Namiki *et al*., 2003). A GST-MDA-7 fusion protein was expressed and purified from a bacterial expression system to generate antigen for producing antibodies recognizing MDA-7/IL-24 protein. In the course of these experiments, analysis was also performed to determine if this purified protein had biological activity. These studies which are described in the following sections, contrasts with reports of activity of purified MDA-7/IL-24 protein expressed and purified by three independent groups using other expression systems (Caudell *et al*., 2002; Parrish-Novak *et al*., 2002; Ramesh *et al*., 2003). Our results confirm that signaling events leading to susceptibility to GST-MDA-7-induced apoptosis are p38MAPK-dependent in transformed cell-specific killing. Data reported here indicate that GST-MDA-7 is taken up by cells and can internalize. Moreover, all pancreatic cancer cells tested with GST-MDA-7 protein are directly susceptible to killing, whereas these cells display resistance to killing following infection by Ad.*mda*-7 (Su *et al*., 2001). In these contexts, the GST-MDA-7 fusion protein provides a valuable reagent for analysing the molecular basis of cancer-specific apoptosis-inducing properties of this novel molecule, which previously was a property restricted to delivery by adenoviral expression vectors. In addition, the ability of GST-MDA-7 to induce cancer cell killing in specific cancer cells, whereas adenovirus mediated *mda*-7/IL-24 does not induce this property, offers potential to increase the therapeutic efficacy of this intriguing clinically relevant cytokine.

Results

GST-MDA-7 fusion protein displays cancer cell-killing specificity and is able to distinguish between transformed and normal cells

The immortalized prostate epithelial cell line P69 and the prostate tumor cell line DU-145 were treated under identical conditions to determine the effect and specificity of GST-MDA-7 protein. After determining the appropriate range of protein needed for activity (data not shown), cells were treated with a predetermined optimum protein concentration (50 ng/μl) and observed daily until cytotoxicity was apparent, which occurred between 48 and 72 h post-treatment compared to cells treated with unfused control GST protein (Figure 1a). Minimal or no cytotoxicity was observed in GST treated normal- or tumor-derived cells. In comparison, significant cell killing was evident in the human prostate cancer cell line, DU-145, after treatment with GST-MDA-7 protein, which had little effect on resistant P69 cells. From these results, it appears that the bacterially derived protein has comparable properties, in terms of cancer cell-specificity, to protein derived from Ad.*mda*-7.

To confirm that the observed cell killing, following treatment with GST-MDA-7 resulted from induction of apoptosis, FACS analysis was performed on susceptible DU-145 prostate cancer and resistant P69 cells. Cells were treated in parallel with GST and GST-MDA-7 protein at 50 ng/μl after plating at 2×10^6 cells/6-cm dish utilizing one plate for each time point. Samples were withdrawn at various time points and fixed in 80% ethanol after trypsinization. Cells were stained with propidium iodide and FACS analysis was performed to determine the proportion of apoptotic cells in the population as a function of time (Figure 1b and c). Treatment of P69 cells up to 96 h with GST-MDA-7 gave a similar FACS profile as untreated or GST control treated cells (Figure 1b), while DU-145 treated cells showed a 50% A_0 population at 96 h compared to less than 21% for untreated and GST-treated populations (Figure 1c). The extent of cell killing is likely underestimated since a certain proportion of cells were highly fragmented or lysed following GST-MDA-7 treatment and could not be analysed by FACS. A certain proportion of dead cells were observed in the untreated and GST-treated samples at later time points due to confluence and cell overgrowth. These data indicated that susceptible cells treated with GST-MDA-7 undergo apoptotic death.

Annexin V staining was performed on susceptible prostate cancer cell lines and resistant normal immortal P69 cells. Annexin V staining confirmed increases in early apoptotic cells (24 h) as a function of GST-MDA-7 treatment in PC-3, DU-145 and LNCaP cells (Figure 1d). In contrast, no significant change in early apoptotic cells was apparent in P69 cells using the same experimental protocol.

GST-MDA-7 induces apoptosis in mutant cell lines defective in the JAK/STAT pathway

We investigated the specific requirements of the JAK/STAT pathway in GST-MDA-7-mediated killing by employing cell lines functionally deficient for JAK/STAT. These included a human fibrosarcoma cell line 2f TGH (parental) and corresponding mutant cell lines derived from it, including U1A (lacking Tyk2), U3A (IFN-unresponsive, lacking STAT1), and U4A (lacking

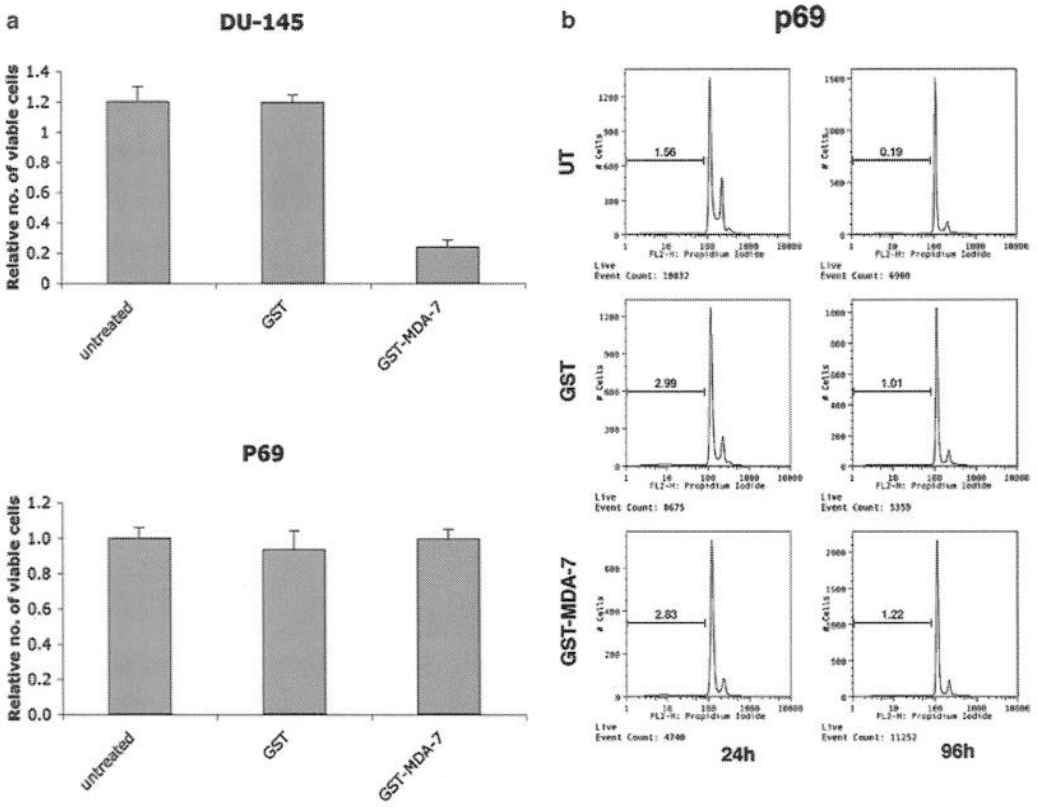


Figure 1 Comparative growth-inhibition and apoptosis-induction in human prostate cells treated with GST or GST-MDA-7. (**a**) Growth inhibition in prostate cell lines: Cells were treated with GST or GST-MDA-7 (50 ng/μl) and cell viability was determined by the MTT proliferation assay 5-days later. Numbers represent a ratio of specific treatments indicated versus untreated cells. An average of three independent experiments is shown $\pm$s.d. (**b**, **c**) Apoptosis induction in prostate cancer cells: cells were treated as described in Materials and methods and the percentage of cells in the A_0 (hypodiploid) fraction (M1 bars) was determined for (**b**) P69 cells for 24 and 96 h and for (**c**) DU-145 cells for 24, 48, 72 and 96 h. Fixation was followed by staining with PI and quantitating by FACS analysis using CellQuest software (Becton Dickinson) as described in Materials and methods. (**d**) Annexin V binding assay. The indicated cell types were treated with GST or GST-MDA-7 (50 ng/μl), stained with FITC labeled Annexin V and PI and immediately analysed by flow cytometry. The percentage of early apoptotic cells was calculated using CellQuest software (Becton Dickinson, San Jose, CA, USA)

JAK1). The human prostate cancer cell PC-3, that does not express STAT3 (Spiotto and Chung, 2000) was used to complete the known spectrum of *mda-7/IL-24*-mediated pathway components. These cell lines were treated with GST-MDA-7 and viability was analysed by using an MTT cell proliferation assay. In addition, two inhibitors of tyrosine kinases, Genistein and tyrphostin AG18 as well as the JAK-selective inhibitor, AG490, were utilized (Sauane *et al*., 2003a). All of these cell lines were susceptible to GST-MDA-7 (Figure 2a, compare GST to GST-MDA-7). These data provide an independent means of confirmation that the activation of JAK/STAT induced by *mda-7/IL-24* can be separated from cell apoptosis induced after GST-MDA-7 treatment, since the cells tested have inactivating mutations in JAK/STAT signaling components. DU-145 cells were also treated with GST-MDA-7 protein during different periods of time and analysed for activation of the JAK/STAT pathway by determining the extent of STAT3 phosphorylation. As evident in Figure 2b, treatment with purified GST-MDA-7 protein does not induce phosphorylation of STAT3, whereas treatment with fibroblast interferon (1000 U/ml) induces a temporal induction of STAT3 phosphorylation.

Cytotoxicity of bacterial GST-MDA-7 protein is inhibited by treatment of cells with the p38MAPK inhibitor SB203580

As previously reported, inhibition of the p38MAPK pathway by pharmacological inhibitors or dominant-negative adenovirus blocked the ability of Ad.*mda-7* to kill different cancer cell types, including PC-3 (Sarkar *et al*., 2002b). PC-3 human prostate cancer-derived cells were incubated in the absence or presence of SB203580 (2 μM), 24 h before treatment with GST or GST-MDA-7 protein and the extent of cytotoxicity was determined (Figure 3a and b). Under these experimental conditions, a significant inhibition of GST-MDA-7-induced killing of PC-3 prostate cancer cells was obtained following

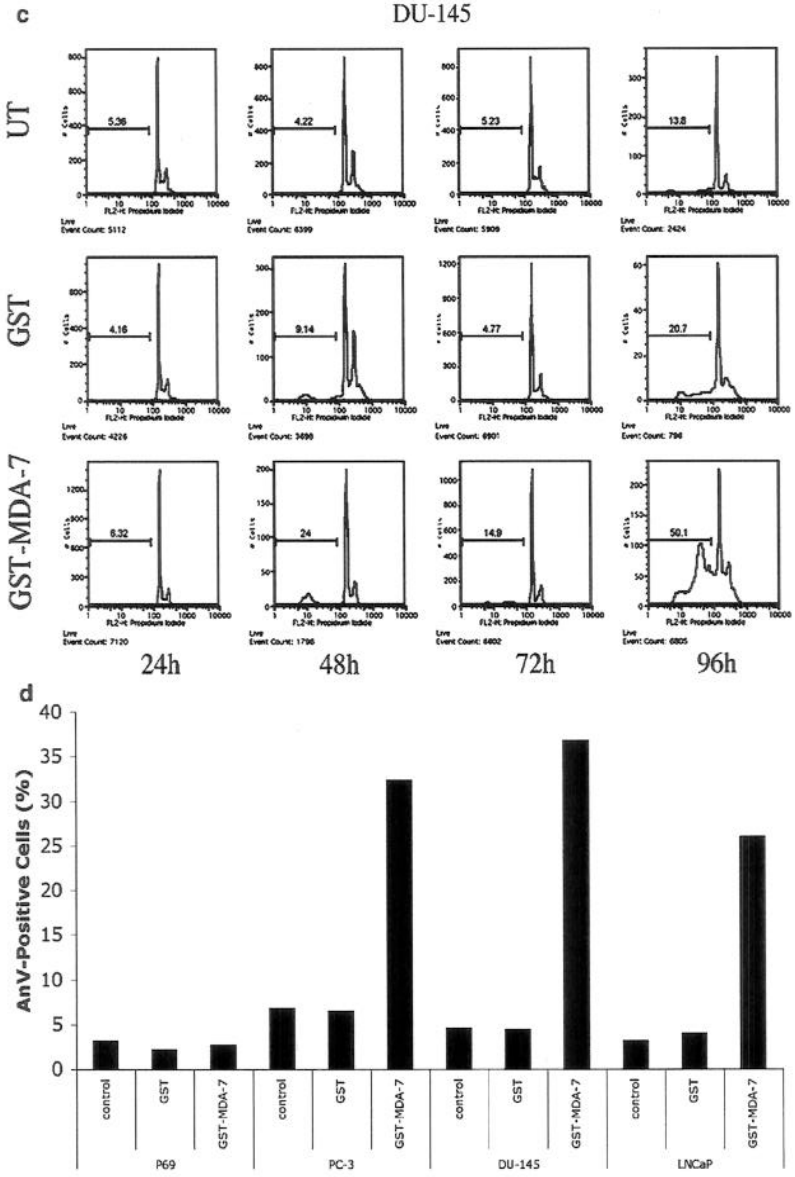


Figure 1 Continued

SB203580 treatment. These results indicate that GST-MDA-7 protein most probably acts in a comparable manner as Ad.*mda-7* by activating the p38MAPK pathway as one of the potential pathways by which transformed cell-specific apoptosis is induced.

GST-MDA-7 protein is able to kill human pancreatic cancer cells in the absence of ablation of Ras signaling by antisense K-Ras

We previously reported that human pancreatic cancer-derived cell lines are not susceptible to direct killing by Ad.*mda-7* (100 PFU/cell) (Su *et al*., 2001). We further showed that lack of killing was due to absence or very low amount of MDA-7/IL-24 protein expression. Treatment of resistant cells with a combination of antisense K-ras (phosphothiorate oligonucleotides or a plasmid expressing this AS molecule) and Ad.*mda-7* vector caused killing which correlated with reappearance of MDA-7 protein. We presently demonstrate that treatment of these resistant cells with purified GST-MDA-7 protein is able to induce cell death directly, strengthening the hypothesis that a translational block might be operational in these and additional MDA-7/

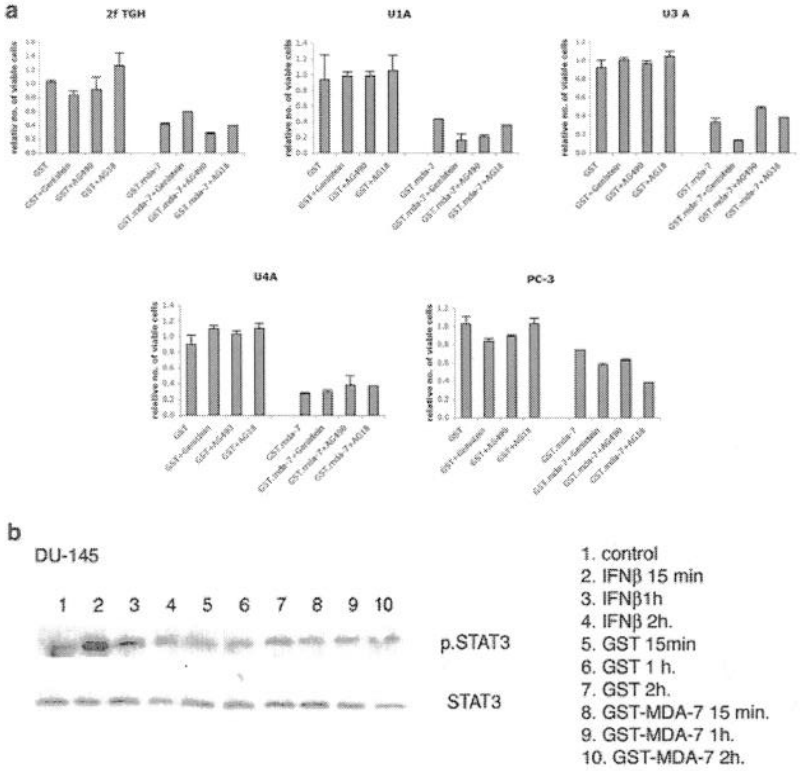


Figure 2 Apoptosis induction by GST-MDA-7 in JAK/STAT-deficient human fibrosarcoma cell lines: (**a**) the indicated cell types were treated with GST or GST-MDA-7 protein. Cells were analysed for cell viability by MTT assay 5-days later. MTT absorbance of untreated control cells was set at 1 to determine relative number of viable cells. Results are the average of three independent experiments ± s.d. (**b**) STAT3 activation in DU-145 cells: protein lysates were collected from uninfected (control) DU-145 cells and after IFN-β (1000 U/ml), GST (50 ng/μl) or GST-MDA-7 (50 ng/μl) treatment from 15 min to 2 h. Samples (50 μg) were run on 10% SDS–PAGE, transferred to a nitrocellulose membrane and probed with rabbit anti-phospho-STAT3 and anti-STAT3 antibodies as described in Materials and methods

IL-24-resistant cells (Figure 4a). Since all of the pancreatic cancer cells treated with GST-MDA-7 protein, irrespective of their K-*ras* status, are directly susceptible to killing, it appears that cells or tumors that appear to be resistant to infection with Ad.*mda*-7, might still be killed following direct exposure to purified protein. This observation suggests added clinical significance in a resistant or uninfectable (by adenovirus) tumor context.

The human pancreatic cancer cell line BxPC-3 is completely resistant to killing by Ad.*mda*-7 either in the presence or absence of K-*ras* ablation (Su *et al*., 2001). This cell line is wild type for *K-ras* and does not express detectable levels of MDA-7 protein after combination treatment with antisense K-*ras* + Ad.*mda*-7. However, exposure of these cells to GST-MDA-7 protein results in cell killing, as shown in Figure 4. Treatment of BxPC-3 and other normal and cancer cell types (not shown) with bacterially expressed and purified GST-MOB-5, the rat orthologue of *mda*-7 (Wang *et al*., 2002), showed no cytotoxic effects on cells and was comparable to treatment with unfused GST protein (Figure 4b). This protein derived from the rat sequence was also a bacterially expressed and purified GST fusion, which had no effect on cell viability under conditions where GST-MDA-7 caused a significant amount of cancer-specific cell death. Therefore, cancer-cell specificity of *mda*-7/IL-24 is further strengthened following parallel utilization of a closely related molecule mob-5 (~80% similar) derived from rat.

Apoptosis induction by exogenously administered GST-MDA-7 protein is likely due to rapid uptake by cells

DU-145 cells were treated with control GST or GST-MDA-7 fusion proteins following plating on coverslips. Cells were washed thoroughly with PBS to remove externally bound material before fixation. Parallel sets of samples were reacted with anti-GST and anti-MDA-7 antibody followed by Alexa Fluor 488 tagged secondary antibody for immunofluorescent detection of protein (Figure 5). Several time points were utilized to determine kinetics of protein uptake and additional controls including secondary antibody alone, and nonsusceptible FM-516-SV normal immortal human melanocytes were used in parallel (not shown). When analysed with anti-

GST antibody, both control GST protein as well as GST-MDA-7 were visualized inside cells in extranuclear locations. Samples processed in parallel, but reacted with anti-MDA-7 specific antibody, indicated an intracellular localization of GST-MDA-7 protein. Specificity is demonstrated due to lack of detection of protein in GST-treated samples reacted with anti-MDA-7 antibody. It therefore appears that both GST as well as

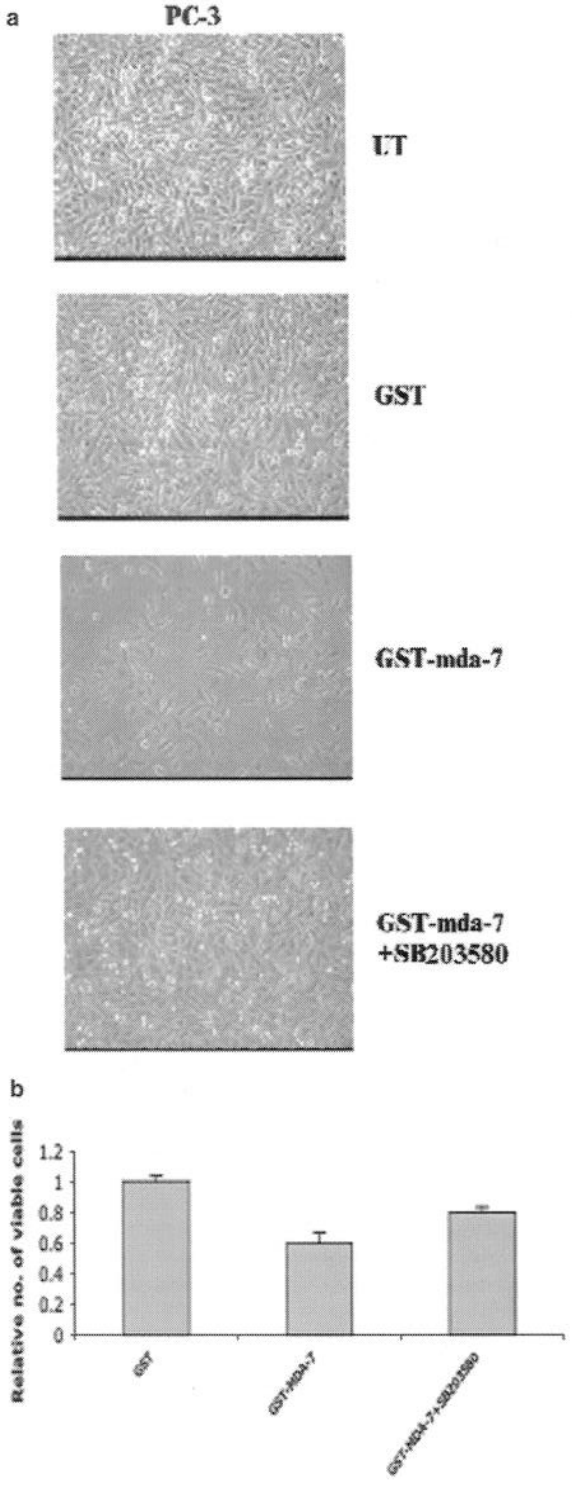
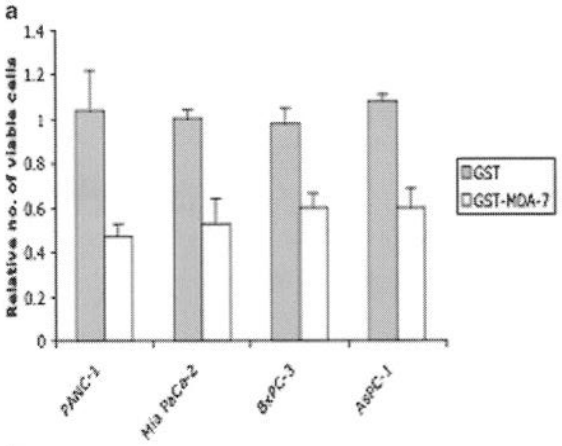


Figure 3 Effect of the p38MAPK inhibitor on GST-MDA-7-induced killing in prostate cancer cell lines: cells were incubated in the absence or presence of SB203580 (2 μM) before treatment with GST or GST-MDA-7. (**a**) Photomicrograph demonstrating cytotoxicity of GST-MDA-7 and the ability of SB203580 to block this effect in PC-3 cells. (**b**) Effect of GST and GST-MDA-7 alone or in combination with SB203580 on growth of PC-3 cells. Cell viability was determined by MTT assay 5-days after infection. MTT absorbance of untreated control cells was set at 1 to determine relative number of viable cells. Results shown are an average of three independent experiments, $\pm$s.d



b

Experimental condition	Cells no.
Untreated	$8.3 \times 10^{4*}$
GST	$8.9 \times 10^{4*}$
GST-MDA-7	$5.9 \times 10^{4*}$
GST-mob5	$10.5 \times 10^{4*}$

*S.D.< 5%

Figure 4 Comparative growth inhibition and apoptosis induction in pancreatic cancer cells treated with GST or GST-MDA-7. (**a**) Cells were treated with GST or GST-MDA-7 and cell viability was determined by the MTT proliferation assay 5-days later. Numbers represent a ratio of specific treatments versus untreated cells. An average of three independent experiments is shown $\pm$s.d. (**b**) BxPC3 (wild-type K-*ras*) human pancreatic cancer cell line was plated in triplicate at 1×10^6 cells/6 cm dish. Surviving cells were counted 72 h after treatment with purified GST, GST-MDA-7, and GST-MOB-5 protein expressed and purified under identical conditions. Untreated cells (UT) were used as an additional control. Cell counts were performed using trypan blue dye exclusion to enumerate the number of surviving cells after treatment

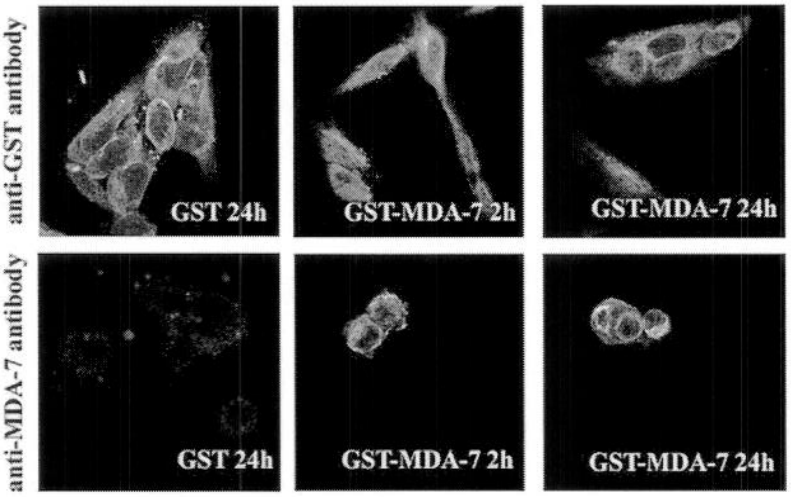


Figure 5 Internalization of GST and the MDA-7 protein, respectively, after treatment with GST or GST-MDA-7: GST and MDA-7 protein localization was analysed by indirect immunofluorescence after treatment of DU-145 cells with purified GST (24 h post-treatment) or GST-MDA-7 protein (2 and 24 h post-treatment). Cells were washed, fixed, and GST or GST-MDA-7 protein, respectively, was detected by indirect immunofluorescence using anti-GST (three panels on top) or anti-*mda*-7/IL-24 antibody (three panels on bottom)

GST-MDA-7 fusion protein are internalized by cells. It is unclear if this is a receptor-mediated process though a more likely possibility is that the GST moiety facilitates uptake of protein by cells, as recently reported (Namiki *et al*., 2003).

MDA-7/IL-24 localizes to the ER/Golgi compartments

Based on previous results indicating that apoptosis induced by *mda*-7/IL-24 have a potent intracellular mode of action and this molecule is active in inducing transformed cell-specific apoptosis even without secretion (Sauane *et al*., 2004; Sieger *et al*., 2004), it was important to determine the subcellular location of GST-MDA-7 protein. Therefore, comparative subcellular localization of MDA-7/IL-24 was analysed in DU-145 cells after treatment with protein for 24 h. GST-MDA-7 protein was detected only in extra-nuclear regions of individual cells. While there was a light background cytoplasmic staining, protein location primarily overlapped that of the endoplasmic reticulum (ER) stained with anti-calreticulin. The colocalization of MDA-7/IL-24 in the Golgi apparatus was also detected via colocalization with anti-GM130 staining. However, no colocalization of MDA-7/IL-24 in mitochondria labeled with MitoTracker red occurred, confirming staining specificity in DU-145 cells (Figure 6). Similar localization results were obtained using the GST-MDA-7 protein in P69 cells, suggesting that differential localization of MDA-7 following treatment with this fusion protein may not be a contributing factor in determining the differential sensitivity of cancer versus normal cells to GST-MDA-7 (unpublished data).

*GST-MDA-7 protein is able to kill human breast cancer cells and it has radiosensitizing effects similar to Ad.*mda-7

Ionizing radiation has been shown to enhance the killing effect of purified GST-MDA-7 protein in glioma cells *in vitro* (Su *et al*., 2003; Yacoub *et al*., 2003b, 2004). To test whether GST-MDA-7 is able to induce a similar effect in breast cancer cells lines and whether radiation can modulate apoptosis, first MDA-MB-231 cells were treated with bacterially synthesized GST-MDA-7 at different concentrations to determine effect on cell growth. As shown in Figure 7a, GST-MDA-7, but not GST, induced a dose-dependent decrease in viability in MDA-MB-231 cells as reflected by MTT assay. MDA-MB-231 cells were treated with bacterially synthesized GST-MDA-7 followed by exposure to ionizing radiation (Figure 7b). GST-MDA-7 suppressed MDA-MB-231 cell growth that was enhanced in a greater that an additive fashion by ionizing radiation (Figure 7b). The direct growth inhibitory effect of purified protein in additional breast cancer cells was observed when MCF-7, T47D and MDA-MB-157 breast tumor cells were treated with the GST-MDA-7 protein (Figure 7c). In contrast, no significant change in viability or growth was observed following treatment of the normal HBL-100 breast epithelial cell line with GST-MDA-7 (Figure 7c). These data provide further support for equivalent cancer-specific cell killing when *mda-7*/IL-24 is applied to cells as a GST-MDA-7 fusion protein produced in bacteria. Moreover, cancer-cell specific killing in breast cancer cells by GST-MDA-7, as previously shown using Ad.*mda-7* (Su *et al*., 1998), occurs in a p53-independent

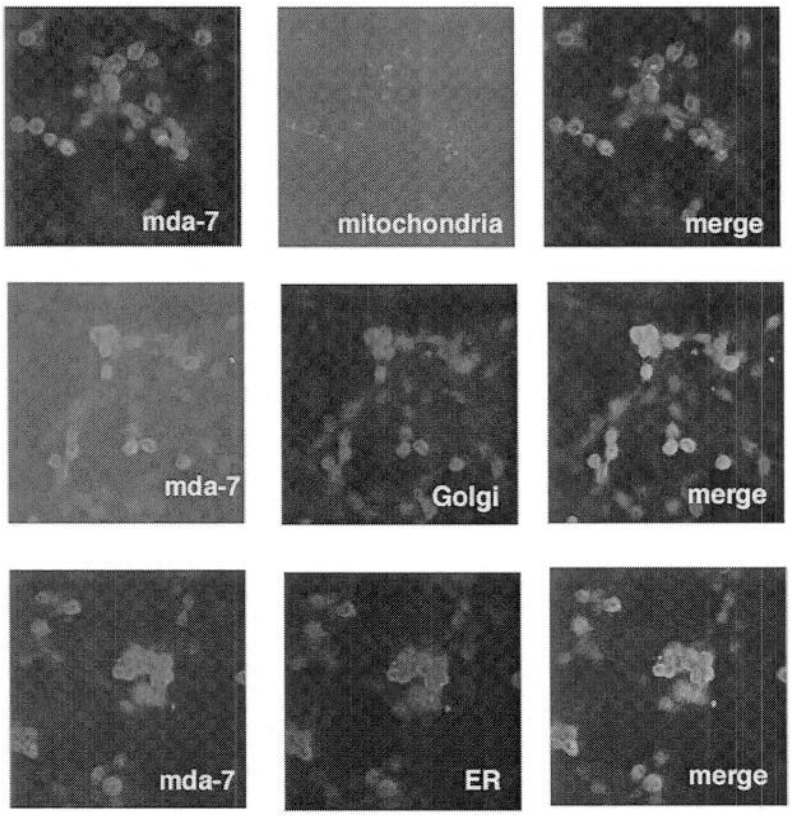


Figure 6 Localization of the MDA-7 protein after treatment with GST-MDA-7: MDA-7/IL-24 protein localization was analysed by indirect immunofluorescence after treatment of DU-145 cells with GST-MDA-7. At 48 h postinfection, cells were fixed and *MDA*-7/IL-24 protein was detected by indirect immunofluorescence using anti-*mda*-7/IL-24 antibody. Images of Golgi, ER and mitochondria were obtained using anti-G130, anti-calreticulin, and MitoTracker, respectively, as described in Materials and methods. Images of the different compartments and *mda*-7/IL-24 were merged to determine extent of colocalization

manner, that is, MCF-7 (is wild-type p53), MDA-MB-157 (is null for p53), and MDA-MB-231 and T47D (are mutant for p53).

Discussion

Considerable interest exists in elucidating the mechanism by which *mda*-7/IL-24-mediated apoptosis differentiates between normal and transformed cells. Precisely, how *mda*-7/IL-24 induces this selective effect is clearly very complex and can vary in a cell-type and cancer-specific context (Sarkar *et al*., 2002a; Fisher *et al*., 2003; Sauane *et al*., 2003b). The antitumor cell activity of the molecule can function independently of JAK/STAT signal transduction pathways that are classically involved in cytokine-mediated activities, now documented using GST-MDA-7 protein as well as previously reported using adenoviral delivery of *mda*-7/IL-24 (Sauane *et al*., 2003a). Additionally, we previously demonstrated by sensitive RT–PCR methodology that apoptosis could be induced in tumor cells not expressing detectable levels of IL-20/IL-22 receptors that bind to MDA-7/IL-24 (Sauane *et al*., 2003a). Moreover, an adenovirus vector expressing a nonsecreted version of MDA-7/IL-24 protein was generated via deletion of its signal peptide, and this nonsecreted protein was as effective as wild-type secreted MDA-7/IL-24 in inducing apoptosis (Sauane *et al*., 2004), confirming that *mda*-7/IL-24-mediated apoptosis can be triggered through intracellular localization as well as via secretion. Our results and recent studies by others clearly reveal that intracellular MDA-7/IL-24 is active in inducing transformed cell-specific apoptosis, probably through mechanisms overlapping and possibly involving pathways associated with ER-stress or the unfolded protein response mechanism (Sauane *et al*., 2004; Sieger *et al*., 2004). Induction of GADD genes is classically associated with the stress response including ER-stress pathways. Induction of GADD genes and further upstream events such as activation of p38MAPK was shown to be induced in a transformed cell-specific

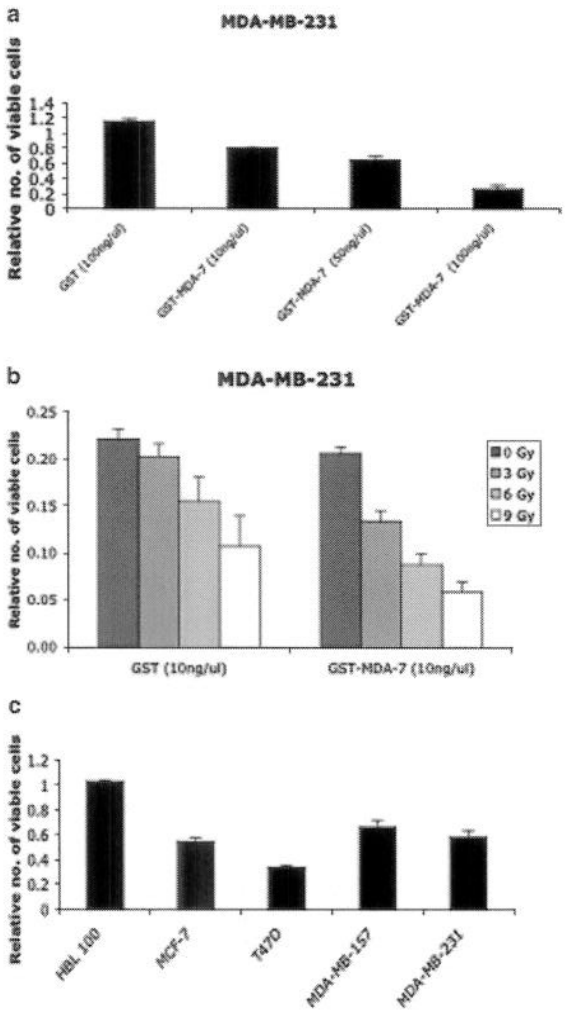


Figure 7 Enhanced radiation-induced cell killing in breast cancer cells treated with GST-MDA-7. (**a**) MDA-MB-231 cells were treated with GST or GST-MDA-7 and cell viability was determined by the MTT proliferation assay 5-days later. Numbers represent a ratio of specific treatments indicated versus untreated cells. (**b**) MDA-MB-231 cells were treated with GST-MDA-7 or GST and 24 h after cells were irradiated (3, 6, 9 Gy). Cells were collected 96 h after irradiation and viability was determined by MTT assay. (**c**) HBL-100, MDA-MB-157, MDA-MB-231, T47D, and MCF-7 were treated with GST or GST-MDA-7 and cell viability was determined by the MTT proliferation assay 5-days later. Numbers represent a ratio of specific treatments indicated versus untreated cells. An average of three independent experiments is shown $\pm$s.d

manner after Ad.*mda-7* infection (Sarkar *et al*., 2002b). Furthermore, Ad.*mda-7* infection upregulated the inositol 1,4,5-trisphosphate receptor (IP3R) in H1299 cells (Mhashilkar *et al*., 2003). IP3R is an intracellular calcium-release channel implicated in apoptosis and localized in the ER. Finally, nonsecreted protein and wild-type secreted MDA-7/IL-24 localizes in the Golgi/ER compartments (Sauane *et al*., 2004), as does the bacterially expressed and purified GST-MDA-7 fusion protein.

Previous studies have documented that secreted MDA-7/IL-24 from supernatants of HEK 293 cells has biological activity. Specifically, in human PBMCs, MDA-7/IL-24 functions as a pro-Th1 cytokine and induces production of IFN-γ, IL-6, and tumor necrosis factor α (Caudell *et al*., 2002). MDA-7/IL-24 is also a potent antiangiogenic effector *in vitro* as well as *in vivo* (Ramesh *et al*., 2003). The studies listed above have likely utilized purified secreted native protein at relatively lower concentrations than achieved via Ad.*mda-7* infection possibly due to limitations of the source of production (a stably transfected 293 cell line, Caudell *et al*., 2002). Purified MDA-7/IL-24 used in these studies was able to activate phosphorylation of STAT1/3. However, none of these studies, utilizing native secreted MDA-7/IL-24 demonstrated growth inhibition or apoptosis induction (Caudell *et al*., 2002; Ramesh *et al*., 2003). Parrish-Novak *et al*. (2002) demonstrated that in NIH:OVCAR-3, MDA-7/IL-24 inhibited cell growth only at doses above 600 pM. This cytostatic effect employs an alternative pathway since STAT1/3 activation was not observed and the authors speculate that it probably occurs through nonclassical receptor activation or might be receptor independent. Further, this growth inhibitory activity was reversible. To try to dissect the pathways involved in the growth inhibitory activity of MDA-7/IL-24 using adenoviral delivery or via purified bacterial GST-fusion protein, it is clear that we need to distinguish between physiological and supraphysiological expression of MDA-7/IL-24 (Fisher *et al*., 2003). In the work by Caudell *et al* (2002), treatment of primary human PMBCs with bacterial or yeast-derived protein required a relatively high amount of protein (μg/ml range) to elicit responses such as secretion of secondary cytokines (IL-6, TNF-α and IFN-γ) compared to subsequent studies described in the same report performed utilizing native MDA-7/IL-24 protein derived from tissue culture supernatants of a stably transfected cell line (Caudell *et al*., 2002). As mentioned above, purified MDA-7 derived-protein from tissue culture supernatant and others, including alternate bacterial (6-Histidine tagged) and baculovirally derived sources have demonstrated cytokine-related activity including receptor binding (Wang *et al*., 2002) and JAK/STAT activation but not transformed cell-specific killing that we have obtained with the GST-fusion described in this report. The reasons for the difference in activity are presently unclear, but might involve factors such as protein stabilization and internalization described below.

Experiments reported here have validated the equivalence of bacterially expressed GST-MDA-7 fusion and adenovirally expressed untagged MDA-7/IL-24 protein in terms of their selective antitumor properties. A significant number of peptides or proteins have been successfully expressed as GST fusion proteins without losing their biological activity (Smith and Johnson,

1988). Studies using GST-driven crystallization have also shown that the final crystal structure of the fused segment remains in their native form (Zhan *et al*., 2001). Like Ad.*mda-7* expressed MDA-7/IL-24 protein, our GST fusion protein can induce killing in cancer-derived cell lines while exerting minimal effects in nontransformed cells (Figure 1) and death occurs via apoptosis as determined by Annexin V staining and FACS analysis, which distinguishes apoptotic from necrotic cell death (Su *et al*., 2003).

In the context of tumor cell killing, GST-MDA-7 also operates in a similar way as does MDA-7/IL-24 via JAK/STAT-independent and MAPK-dependent pathways (Figures 3 and 4) as has previously been demonstrated for the *mda*-7/IL-24 gene product delivered by an adenovirus (Sarkar *et al*., 2002b; Sauane *et al*., 2003a). Furthermore, treatment with GST-MOB-5 (rat orthologue of *mda-7* having ~80% homology (Wang *et al*., 2002), purified under the same conditions, did not induce cytotoxic effects in cells and was comparable to treatment with GST protein, indicating that the observed apoptotic effects are not attributed to the novel modifications of the fusion protein but rather are likely a consequence of the activity of the MDA-7/IL-24 moiety of the fusion. The GST-tag might, however, contribute to both stability as mentioned above as well as facilitation of protein uptake. There is also a possibility that the uptake process is receptor mediated but the likelihood of currently recognized cognate *mda-7*/IL-24 receptor participation in this process is not very strong. In general, the data shown here indicate that GST-MDA-7 is functionally equivalent to native MDA-7/IL-24 protein with respect to its tumor-killing attributes.

Pancreatic cancer is one of the most lethal forms of all malignancies, with the lowest probability of survival (Blaszkowsky, 1998; Regine *et al*., 1998; Hilgers and Kern, 1999; Lorenz *et al*., 2000; Rosenberg, 2000). Treatment with Ad.*mda-7* alone has no effect on these tumor cells despite the pervasive antitumor toxicity of MDA-7/IL-24 protein, and it was postulated that this resistance is due to interference of *mda-7/IL-24* translation or protein stability by the mutant K-*ras* pathway present in 85–95% of tumors (Su *et al*., 2001). While MDA-7/IL-24 protein was not detected after infection with Ad.*mda-7*, combined infection with antisense K-*ras* (phosphorothioate oligonucleotides or an antisense K-*ras* expression construct) in pancreatic cancer cells resulted in production of MDA-7/IL-24 protein and consequently cell death (Su *et al*., 2001). We demonstrated that direct application of MDA-7/IL-24 proteins in the form of GST-MDA-7 could kill mutant K-*ras* pancreatic cancer cells (Figure 4). Hence, these studies establish that when a sufficient amount of active MDA-7/IL-24 protein is delivered to pancreatic tumor cells, killing is effective and the source of their apparent resistance can be most likely attributed to interference with MDA-7/IL-24 protein translation and/or stability by the K-*ras*-activated pathway.

Cancer gene therapy using Ad.*mda-7* has significant promise and based on initial successes continues to be evaluated in Phase I/II clinical trials (Chada *et al*., 2001; Nemunitais, 2003). The potential use of GST-MDA-7 protein as a therapeutic is intriguing, since it can enlarge the existing MDA-7/IL-24 therapeutic scope to cover tumors resistant to or uninfectable by Ad.*mda-7*. It is presently demonstrated that direct application of GST-MDA-7 can kill pancreatic cancer cell lines previously resistant to Ad.*mda-7* (Figure 4). Another example is renal cell carcinoma (RCC), where recent studies demonstrated that Ad.*mda-7* did not affect RCC proliferation due to weak infectivity, but GST-MDA-7 caused a dose-dependent growth suppression (Yacoub *et al*., 2003b, 2004). Additional work has demonstrated that both Ad.*mda-7* as well as the GST-MDA-7 fusion protein is able to radiosensitize primary human glioblastoma cells to comparable extents (Su *et al*., 2003; Yacoub *et al*., 2003a, 2004). When analysed with anti-GST antibody, both control GST protein as well as GST-MDA-7 is visualized inside cells in extranuclear locations. Samples processed in parallel but reacted with anti-MDA-7 antibody show an intracellular localization of GST-MDA-7 protein. Specificity is demonstrated due to lack of detection of protein in GST-treated samples reacted with anti-MDA-7 antibody. It therefore appears that GST as well as GST-MDA-7 fusion proteins are internalized by cells. It is unclear if this is a receptor-mediated process, although a more likely possibility is that the GST moiety facilitates uptake of protein by cells as recently reported (Namiki *et al*., 2003).

The retention of biological activity and particularly, cancer cell specificity of bacterially produced GST-MDA-7 was unexpected given the large size of the amino-terminally located tag and its likely interference in receptor binding. However, our recent findings that the *mda*-7/IL-24 gene functions efficiently in the absence of secretion following mutation of its signal peptide (Sauane *et al*., 2004) and the ability of GST-fusion proteins to enter cells could explain why GST-MDA-7 demonstrates cancer-cell-specific apoptosis inducing activity. In these contexts, the availability of this bioactive fusion protein allows for more detailed kinetic studies of MDA-7/IL-24 action, such as varying exposure time, which is difficult to achieve with Ad.*mda-7*. The purified active protein can provide an additional reagent of some utility in the ongoing analysis and characterization of *mda-7/IL-24* as a potential cancer gene therapeutic. Accordingly, the observation that purified GST-MDA-7 protein is able to kill Ad.*mda-7*-resistant pancreatic cancer cells is particularly relevant. This indicates potential complementarity in utilization of two distinct reagents (Ad.*mda-7* and GST-MDA-7) for analytical and therapeutic uses. Additionally, now having this unique combination of reagents in hand to deliver *mda*-7/IL-24 provides an unparalleled opportunity for defining the biochemical and molecular events controlling cancer-cell specificity of this clinically pertinent cytokine.

Materials and methods

Cell lines and culture conditions

PC-3, LNCaP and DU-145 (human prostate cancer), 2f TGH (human fibrosarcoma) and corresponding mutant sublines U1A, U3A, U4A and U5A (Darnell *et al*., 1994) (kind gift of G Stark, Cleveland Clinic, OH, USA) were grown in Dulbecco's modified Eagle's medium/F12 (DMEM/F12) supplemented with 10% FBS at 37°C in a humidified 5% CO_2 incubator. Human pancreatic carcinoma cell lines (AsPC-1, BxPC-3, MIA PaCa-2, and PANC-1) were maintained in RPMI 1640 medium containing 10% FBS, antibiotics and L-glutamine (Blumberg *et al*., 2001). Normal human breast epithelial cells and human breast cancer-derived lines MCF-7, T47D, MDA-MB-231, and MDA-MB-157 were grown in DMEM containing 10% FBS.

Synthesis of GST-MDA-7

Standard cloning procedures were used to generate a bacterial expression vector comprising in-frame fusion of the *mda-7*/IL-24 open reading frame 3′ to the GST open reading frame in GST-4T2 vector (Amersham Pharmacia, NJ, USA), using *Bam*H1 and *Not*1 sites introduced into the *mda-7*/IL-24 ORF by PCR. Expression of protein was performed by inoculating an overnight culture at 1 : 100 dilution followed by incubation at 25°C until an A_{600} of 0.4–0.6 nm was reached, followed by induction with 0.1 μM isopropyl-1-thio-β-D-galactopyranoside (IPTG) for 2 h. Cells were harvested by centrifugation and sonicated in PBS followed by centrifugation to obtain soluble protein. The lysates were bound to a glutathione-agarose column (Amersham Pharmacia, NJ, USA) at 4°C for 2 h followed by washing with 50 volumes of PBS and 10 volumes of PBS with 500 mM NaCl. Passing 20 mM-reduced glutathione through the column and collecting 1 ml fractions performed elution of bound protein. Fractions were analysed by gel electrophoresis, and positive samples were dialysed against 1000 volumes of PBS for 4 h with one change, followed by 500 volumes of DMEM for 4 h. Protein concentration was estimated by Bradford assays, as well as gel electrophoresis, in conjunction with Coomassie blue staining. Samples were tested for activity using GST protein as control. An antipeptide, rabbit polyclonal antibody was raised to specifically detect MDA-7 protein and was used in these studies at 1 : 1000 dilution for immunoblotting and 1 : 200 dilution for immunofluorescence.

MTT assays

Cells were plated in 96-well dishes (1×10^3 cells/well) in DMEM/F12 containing 10% FBS and allowed to attach for 12 h prior to GST or GST-MDA-7 treatment, usually at 25–50 ng/μl. Treatment with inhibitors was initiated 1 h before treatment with protein. During a 5–7-day-treatment period, the medium was changed twice with fresh inhibitor containing medium at days 3 and 6. Cell growth and viable cell numbers were monitored by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) staining as described (Lebedeva *et al*., 2000, 2002). The resulting absorbance measured at 595 nm is directly proportional to the number of viable cells.

Annexin-V binding assay

Cells were trypsinized and washed once with complete media. Aliquots of the cells (5×10^5) were resuspended in complete medium (0.5 ml) and stained with FITC-labeled Annexin-V (kit from Oncogene Research Product, Boston, MA, USA) according to the manufacturer's instructions. Propidium iodide (PI) was added to the samples after staining with Annexin-V to exclude late apoptotic and necrotic cells. The FACS assay was performed immediately after staining.

FACS analysis

Cells were trypsinized and washed once with complete media. Aliquots of cells (5×10^5) were resuspended in complete medium (0.5 ml). Propidium iodide (PI) was added to the samples. FACS assays were performed immediately after staining. The percentage of cells in the apoptotic (A_o) fraction was calculated using CellQuest software (Becton Dickinson).

Western blot analyses

Cell lines were grown on 10-cm plates and protein extracts were prepared with RIPA buffer containing a cocktail of protease inhibitors. A total of 50 μg of protein was applied to 12% SDS–PAGE and transferred to nitrocellulose membranes. The membranes were probed with polyclonal antibodies to MDA-7/IL-24, phospho-STAT3 and total STAT3 (Cell Signalling Technology, MA, USA).

Immunofluorescence analyses

DU-145 cells were grown in chamber slides (Falcon-BD, CA, USA) fixed with 2% paraformaldehyde, permeabilized by 0.1%. Triton X-100, and then incubated with primary antibodies: anti-rabbit *mda-7*, GM130 (BD Pharmingen, CA, USA), LAMP1/2 (Santa Cruz, CA, USA), Calreticulin (BD Pharmingen, CA, USA) and Mitrotrack marker (Molecular Probes, Eugene, OR, USA). FITC-conjugated donkey anti-mouse IgG and anti-rabbit IgG (Molecular Probes, Eugene, OR, USA) were used for visualization on a Zeiss LSM 510 fluorescence microscope.

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